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Microbiological Treatment of Acid Mine Drainage Waters



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16. ABSTRACT: Laboratory tests demonstrated the ability of acidophilic iron bacteria to oxidize ferrous iron in acid mine drainage waters to prepare such waters for relatively inexpensive limestone neutralization. Iron bio-oxidation rate was determined to be directly proportional to iron concentration for ferrous iron levels between 10 and 9000 mg/l. Oxidation was also shown to be temperature dependent with the most rapid oxidation generally occurring between 20 and 30°C and decreased rates occurring at either higher or lower temperatures. The reaction required aeration rates of 1/100 VVM to support microbial oxidation of 500 mg/l ferrous iron, between 1 and 10 mg/l ammonia to support microbial oxidation of 800 mg/l of ferrous iron, and between 0.55 and 5.5 mg/l of phosphate to support microbial oxidation of 9000 mg/l of ferrous iron. Series multistaging of reaction vessels offered significant operational efficiency over a single stage. Although sulfate reducing bacteria were present in all samples, they neither grew nor produced H₂S at pH below 5.5. Significant scale-up problems were encountered in going from a 1 1/2 gallon laboratory scale system to a 1000 gallon pilot plant oxidation vessel for reasons as yet unknown. (Lowry-Texas)

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by

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from page 5

ABSTRACT

Laboratory studies demonstrated that both pure cultures and fresh field cultures of acidophilic iron bacteria could readily oxidize ferrous iron in both synthetic and natural acid mine drainage waters. In the presence of adequate forms and amounts of oxygen, carbon dioxide, nitrogen and phosphorus, ferrous iron was oxidized at rates up to 600 mg/l/hr. Average oxidation rate was found to vary with the ferrous iron concentration in the water; i.e., the higher the ferrous iron content, the greater the oxidation rate. Approximate requirements of oxygen, carbon dioxide, nitrogen and phosphorus by the iron bacteria were established.

Multistaging of oxidation vessels in series was found to produce a more effective microbial oxidation system than use of a single oxidation reservoir.

Limestone neutralizations of partially oxidized acid mine waters showed that such waters containing up to 90 mg/l ferrous iron could be successfully neutralized and result in discharge waters containing ≤ 7 mg/l total iron. *were conducted*

For reasons as yet undetermined, attempts to duplicate laboratory findings with a 2,000-gallon pilot plant were not completely successful.

Although sulfate-reducing bacteria were isolated from all of nine acid mine discharges examined, attempts to grow the cultures or produce hydrogen sulfide at pH values below 5.5 were unsuccessful.

This report was submitted in fulfillment of Grant 14010 ENW between the Environmental Protection Agency and Continental Oil Company.

Key Words: Acid mine water, bacteria, iron bacteria, sulfate-reducing bacteria, limestone, oxidation of ferrous iron, oxidation rate, neutralization, pilot plant

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SECTION I

CONCLUSIONS

1. Acidophilic iron bacteria can be used successfully to oxidize ferrous iron in acid mine drainage waters to prepare such waters for relatively inexpensive limestone neutralization.
2. At ferrous iron concentrations between 10 and 9,000 mg/l, the rate of iron bio-oxidation is significantly dependent on ferrous iron concentration. High oxidation rates occur at high iron concentrations and low rates at low iron concentrations.
3. Series multistaging of microbial oxidation vessels offers operational efficiency over a single oxidation vessel.
4. To achieve a ferrous iron level of about 100 mg/l the following oxidation treatment periods are required:

Influent Fe ⁺⁺ (mg/l)	Stages	Total Residence Time (hours)
9,000	1	87
9,000	2	18
1,000	1	4
500	1	4

5. Bacterial oxidation of ferrous iron is most rapid at 20 - 30°C with decreased rates occurring at higher or lower temperatures. An exception is a species of Thiobacillus which oxidizes iron equally well at 40°C.
6. Aeration rates of 1/100 VVM appear to be adequate for microbial oxidation of mine water containing 500 mg/l ferrous iron.
7. Between 1 and 10 mg/l ammonium ion is required to support microbial oxidation of 800 mg/l ferrous iron. Nitrate or nitrite can serve as the nitrogen source.
8. Between 0.55 and 5.5 mg/l of phosphate is needed to support microbial oxidation of 9,000 mg/l ferrous iron.
9. Significant scale-up problems were encountered in going from a 1 1/2-gallon bench-size microbial oxidation system to a 1,000-gallon pilot plant oxidation vessel.
10. Although sulfate-reducing bacteria are present in acid mine drainage water, they will not grow or produce H₂S at pH values below 5.5.

SECTION II

RECOMMENDATIONS

Microbial Oxidation of Ferrous Iron

1. The problems encountered in scale-up from laboratory-size systems to pilot plant units should be more thoroughly investigated.
2. Other types of new waste treatment concepts, in particular those having large surface contact areas, should be examined as microbial oxidation systems for mine drainage water.
3. Pilot plant microbial treatment studies of mine drainage waters should include limestone neutralization and settling steps so that a complete economic analysis of the process can be ascertained.

Removal of Iron with Microbially Produced H_2S

1. Since sulfate-reducing bacteria will not grow at pH values below 5.5, use of these organisms downstream of the neutralization step should be investigated.
2. A search should be made for a suitable, inexpensive carbon source that will allow the rapid propagation of sulfate-reducing bacteria in neutralized mine discharge waters.

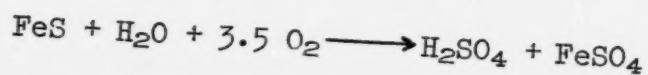
SECTION III

INTRODUCTION

The purpose of this study was to determine if the abilities of certain bacteria to oxidize ferrous iron or to convert sulfate to hydrogen sulfide could be applied to the neutralization and subsequent removal of iron from difficult-to-treat mine drainage waters. If one or both of these concepts could be successfully utilized, the expense of adequately treating these types of problem waters might be significantly reduced.

In certain sections of the country mine drainage waters are one of the principal contributors to water pollution. These discharge waters are characterized by a high dissolved iron content, and many are quite acidic. The problem is particularly acute in the coal mining industry even though modern mining practices have greatly reduced such discharges from operating mines. Recent estimates indicate that from 60 to 90 percent of the total mine drainage water is emitted from worked out or abandoned mines (1,2). The quantity of mine drainage in the state of Pennsylvania alone is 5 to 10 billion gallons per day (3).

The acidity and iron content of mine drainage waters originate from pyrites present in the coal seams and surrounding strata. In the presence of water (surface water and underground aquifers) and oxygen, pyrite is oxidized to sulfuric acid and ferrous sulfate which are carried away by the water.



Although some success in preventing this reaction from occurring can be achieved by water diversion and maintenance of a reducing environment, the complexities of mining and the abandoned mine problem make it seem likely that treatment of acid mine drainage waters will remain a significant problem for the foreseeable future.

Numerous concepts for removing the undesirable iron and acidity from mine discharge waters have been investigated, and some are listed below:

- Ion Exchange (4)
- Reverse Osmosis (5)
- Activated Carbon Catalysis (6)
- Foaming (Addition of a Surfactant) (7)
- Freezing (8)
- Addition of Hydrogen Sulfide Gas (9)
- Production of Microbial Polymer Adsorbents (10)
- Neutralization Followed by Aeration (11)

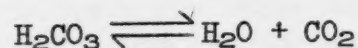
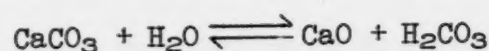
Except for special circumstances, poor efficiencies or adverse economics appear to rule out the present use of most of these techniques.

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The most widely used treatment for acid mine water at the present time is hydrated lime neutralization. This process typically involves the addition of lime followed by vigorous aeration to oxidize the ferrous hydroxide to the ferric state. The ferric hydroxide is then allowed to settle in a series of holding ponds. The precipitate thus formed has a very slow settling rate and a low compaction factor. Not only are large ponds required to provide ample settling time but also are necessary to accommodate the voluminous amount of iron sludge produced. Although the final effluent from such a system is acceptable for surface stream discharge, the problem of sludge recovery, dewatering and disposal is a formidable one (12).

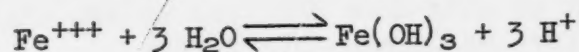
While much less expensive limestone can be successfully used to treat acid discharge waters containing ferric iron or waters having low concentrations of ferrous iron, the more prevalent acid mine discharges that contain relatively high concentrations of ferrous iron are not directly amenable to limestone treatment.

When limestone (calcium carbonate) is added to water bearing an acidic pH, the following reactions occur:



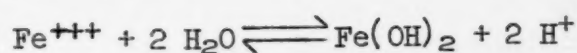
Carbon dioxide is released and acidity is neutralized. While these reactions proceed readily at acidic pH values, calcium carbonate neutralization cannot efficiently take the pH to much above 6.

Ferric hydroxide is formed by ferric iron salts in the presence of water.



Ferric hydroxide becomes water insoluble at about pH 5.5; thus limestone neutralization of acid mine water bearing ferric iron will result in the precipitation of ferric hydroxide. Ferric hydroxide precipitates rapidly and produces a relatively small amount of sludge which has a relatively high solids content.

Ferrous salts in water also hydrolyze.



However, ferrous hydroxide is soluble in water up to pH 9-10. Since calcium carbonate cannot elevate the pH to this region, limestone neutralization will not allow the precipitation of ferrous hydroxide (13).

Thus, in order to realize the obvious economic benefits from using limestone instead of hydrated lime to neutralize acid ferrous mine drainage

waters (limestone costs about one third as much as hydrated lime), it is necessary that the iron present be converted to the ferric state prior to neutralization. Even in the presence of oxygen, this oxidation process is extremely slow at low pH values. For example, at pH 3.5 the half time of this reaction in water saturated with oxygen is 2,000 days (14).

The existence of certain bacteria, commonly known as the acidophilic iron bacteria, that specialize in the oxidation of ferrous to ferric iron at low pH values has been known for many years (15). In fact, these microorganisms have been isolated from many mine discharge waters. Acidophilic iron bacteria are autotrophic (do not require organic substances for growth) and are classified as the Thiobacillus-Ferrobacillus group. These bacteria utilize carbon dioxide as their source of carbon and oxidize ferrous iron to the ferric state in order to obtain energy to drive their cell machinery. In addition to carbon dioxide, oxygen and ferrous iron, these organisms also require lesser quantities of nitrogen and phosphorus and trace amounts of other minerals. It has not been demonstrated, however, that this microbial catalytic activity can be economically utilized to satisfactorily prepare acid ferrous mine drainage water for limestone treatment.

While the use of hydrogen sulfide gas has been examined as a technique for the precipitation and removal of iron in mine drainage water as iron sulfide, the value of commercial hydrogen sulfide has made this concept relatively unattractive (9). However, mine discharge waters are not lacking in sulfate and certain bacteria, in particular Desulfovibrio desulfuricans, are capable of prolific hydrogen sulfide production from sulfate. If this process could be shown to operate in mine drainage waters, using a cheap carbon source to satisfy the carbon requirements of the organisms, an inexpensive technique for iron removal from these discharges would result.

SECTION IV

METHODS

MICROBIAL OXIDATION OF IRON

Iron Bacterial Cultures

Acidophilic autotrophic iron bacteria were cultured from six mine drainage water outfalls in southwestern Pennsylvania and northwestern West Virginia. Waters were selected that differed in chemical composition in an attempt to cover the broad spectrum of water types associated with mine drainage. Culture of the iron bacteria from these sources was initiated on location by inoculating 10 ml samples of the mine waters into 90 ml volumes of sterile 9K medium (Table 1) in 4-ounce screw cap bottles. Following transport to the laboratory and incubation at room temperature, 10 ml aliquots from the bottles were used to inoculate 90 ml volumes of sterile 9K medium in 250 ml erlenmeyer flasks. These flasks were incubated at 25°C on a gyrotory shaker operating at 200 rpm and culture ability to oxidize ferrous to ferric iron verified. Acidophilic autotrophic iron bacterial cultures were obtained in this manner from all of the mine drainage outfalls samples (see top section of Table 2). Viable stock cultures were maintained throughout the study by transferring every four days into fresh 9K medium.

In addition to these field cultures of acidophilic iron bacteria, pure cultures of Thiobacillus ferrooxidans, Ferrobacillus ferrooxidans and Ferrobacillus sulfooxidans were obtained through the courtesies of Drs. J. V. Beck, D. G. Lundgren, W. W. Leathen and P. R. Dugan. These cultures were maintained in the same manner as described for the field cultures and are also listed in Table 2.

Iron Analysis

Ferrous iron and total iron analyses were performed using the p-diphenylamine sulfonic acid - dichromate titration technique (16).

Media and Culture Methods

The basic synthetic medium used throughout the study was the 9K medium of Silverman and Lundgren (17). The synthetic medium was used for most of the investigations to provide reproducibility and to eliminate the problems of transportation and instability associated with natural mine drainage waters. For studies that required the utilization of mine discharge waters, the waters were collected and maintained at ice bath temperature until used.

All of the bacterial growth and oxidation studies were carried out in 250 ml or 2,000 ml erlenmeyer flasks containing 100 ml or 1,000 ml of

TABLE 1
COMPOSITION OF 9K IRON BACTERIAL MEDIUM

$(\text{NH}_4)_2\text{SO}_4$	3 g
KCl	0.1 g
K_2HPO_4	0.5 g
$\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$	0.5 g
$\text{Ca}(\text{NO}_3)_2$	0.01 g
H_2SO_4 (10 N)	1 ml
$\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$ (14.74% wt/vol solution)	300 ml
Distilled Water	700 ml
Final pH	2.0 - 2.5

TABLE 2

ACIDOPHILIC IRON MICROBIAL CULTURES AND THEIR SOURCES

<u>IRON BACTERIAL CULTURES</u>	<u>SOURCES</u>
FIELD CULTURES	
Southwest-1	Southwest Mine Drainage Outfall*
Southwest-2	Southwest Mine Drainage Outfall*
Arden-1	Arden Mine Drainage Outfall*
Arden-2	Arden Mine Drainage Outfall*
Marchand-1	Marchand Mine Drainage Outfall*
Marchand-2	Marchand Mine Drainage Outfall*
Indian Creek	Indian Creek Mine Drainage Outfall*
Main West	Main West Mine Drainage Outfall*
Lynch Shaft	Lynch Shaft Mine Drainage Outfall*
PURE CULTURES	
<u>Thiobacillus ferrooxidans</u> (Beck)	J. V. Beck, Brigham Young University
<u>Ferrobacillus ferrooxidans</u> (Syracuse)	D. G. Lundgren, Syracuse University
<u>Ferrobacillus ferrooxidans</u> (Leathen)	W. W. Leathen, Mellon Institute
<u>Ferrobacillus sulfooxidans</u> (Leathen)	W. W. Leathen, Mellon Institute
<u>Ferrobacillus ferrooxidans</u> (Dugan)	P. R. Dugan, Ohio State University

* Mine outfalls located in southwestern Pennsylvania and
northwestern West Virginia

<u>SULFATE-REDUCING BACTERIAL CULTURES</u>	<u>SOURCES</u>
Southwest*	Mine Drainage Water
Arden*	Mine Drainage Water
Indian Creek*	Mine Drainage Water
Core*	Mine Drainage Water
Brewer*	Mine Drainage Water
Marchand	Mine Drainage Water
Main West	Mine Drainage Water
Lynch Shaft	Mine Drainage Water
Fetty	Mine Drainage Water
Mid-Continent Strain*	Mine Drainage Water
	API Stock Culture
* Test cultures	

medium or in a continuous culture system that will be described in a subsequent section of this report. Culture flasks were agitated on a gyrotory shaker operating at 200 rpm at a controlled temperature.

Inoculum for flasks or the continuous culture system consisted of a 48-72 hour old shake flask culture either used directly from the flask or as a washed cell suspension. Cell suspensions were prepared by centrifuging the cells from the culture medium, washing two times with pH 2.5 water (distilled water acidified with sulfuric acid) and resuspending the cells in pH 2.5 water. The suspension was used immediately on a 10 percent basis; i.e., cells collected from a 100 ml growth flask were used as inoculum for 1,000 ml of fresh medium.

Cell Population

Direct microscopic counts using a Petroff-Hausser counting chamber were found to be exceedingly difficult to perform because of the presence of inanimate solids (primarily precipitated iron) in the preparations which obscured many of the very small cells. These solids also interfered with the direct microscopic counting technique when the organisms were collected on a membrane filter.

The use of solid 9K medium agar plates for dilution counting was also found to be unacceptable. The principal problem encountered with this method was the very long incubation period required for colonial development.

It was found that serial dilution in tubes of 9K medium could be successfully used to determine the approximate iron bacterial population of a given system. Regardless of whether the tubes were agitated or left undisturbed, high dilutions required seven to eight days of incubation before they could even be read microscopically. Upon further incubation, growth was evidenced by the precipitation of iron from solution in the tubes and development of turbidity. The reciprocal of the highest dilution tube exhibiting these characteristics was taken to represent the number of organisms in the original sample. Although this technique required more than a desirable length of time to determine populations, it was selected for use in studies requiring iron bacterial counts.

Cell Mass

A Hewlett-Packard FM Model 185 CHN analyzer was used to quantitate the amount of carbon present in known volumes of iron bacterial culture. Since 9K medium is inorganic, the carbon present in the very acid culture should represent cellular carbon.

Samples were withdrawn at intervals from growing shake flask cultures and subjected to one of two preparatory procedures for carbon analyses. One technique consisted of collecting the solids present in a 10 ml sample on a tared 0.45 micron membrane filter which was then dried in a vacuum at 65°C to a constant weight. Following careful recovery of the solids

present on the filter, they were subjected to CHN analysis. The other method entailed centrifugal sedimentation of the solids present in a 10 ml sample which were then recovered, dried, weighed and analyzed for carbon content.

The data shown in Figures 1 and 2 illustrate the utility of this concept in following iron bacterial cell mass by organic carbon content of the medium. Although cell mass determinations were not carried out in ensuing studies, it was felt that the success of the technique should be reported.

An attempt to directly measure the organic content of growing culture samples was made utilizing a total organic carbon analyzer. Serious positive interferences by medium constituents were encountered, and no attempts were made to identify or circumvent the interferences.

Continuous Culture System

For continuous culture studies a continuous feed system was constructed using a FABCO-BUSH apparatus (Houston Glass Fabricating Company, Houston, Texas) as the central component. A schematic of the apparatus is shown in Figure 3.

This system was used to more thoroughly investigate the nutritional and environmental factors found to be significant in shake flask studies as influencing microbial oxidation of ferrous iron. Substrate (9K medium or field water) was stored in a reservoir from which it was pumped to the first oxidation stage. Average retention time in this vessel was varied by changing the feed pump rate. The first stage contained a working volume of six liters and was aerated via three fritted glass dispersion tubes located at the bottom of the vessel. The inner cone and center tube were designed primarily to permit control over the solids content of the vessel contents; but since the iron bacteria being used tend to exist as single discrete cells rather than clumps, these system components served only to insure complete mixing of the vessel contents. The second oxidation stage was aerated via a single, coarse-grade gas dispersion tube; and mixing was insured by use of a magnetic stirrer insulated to prevent significant heat transfer. A five-gallon carboy served to collect the final effluent. Reduced pressure on this vessel was used to draw liquid from the first stage into the second stage and then into the effluent collector.

Sulfate-Reducing Bacteria

Nine different mine drainage water outfalls in Pennsylvania and West Virginia were sampled and examined for the presence of sulfate-reducing bacteria. D. desulfuricans (identification based on morphology and hydrogen sulfide production) was found in all nine waters.

Isolation of D. desulfuricans was carried out in the following manner. Sterile 4-ounce screw cap bottles were completely filled with mine drainage water at the outfall site and tightly capped. One ml of the

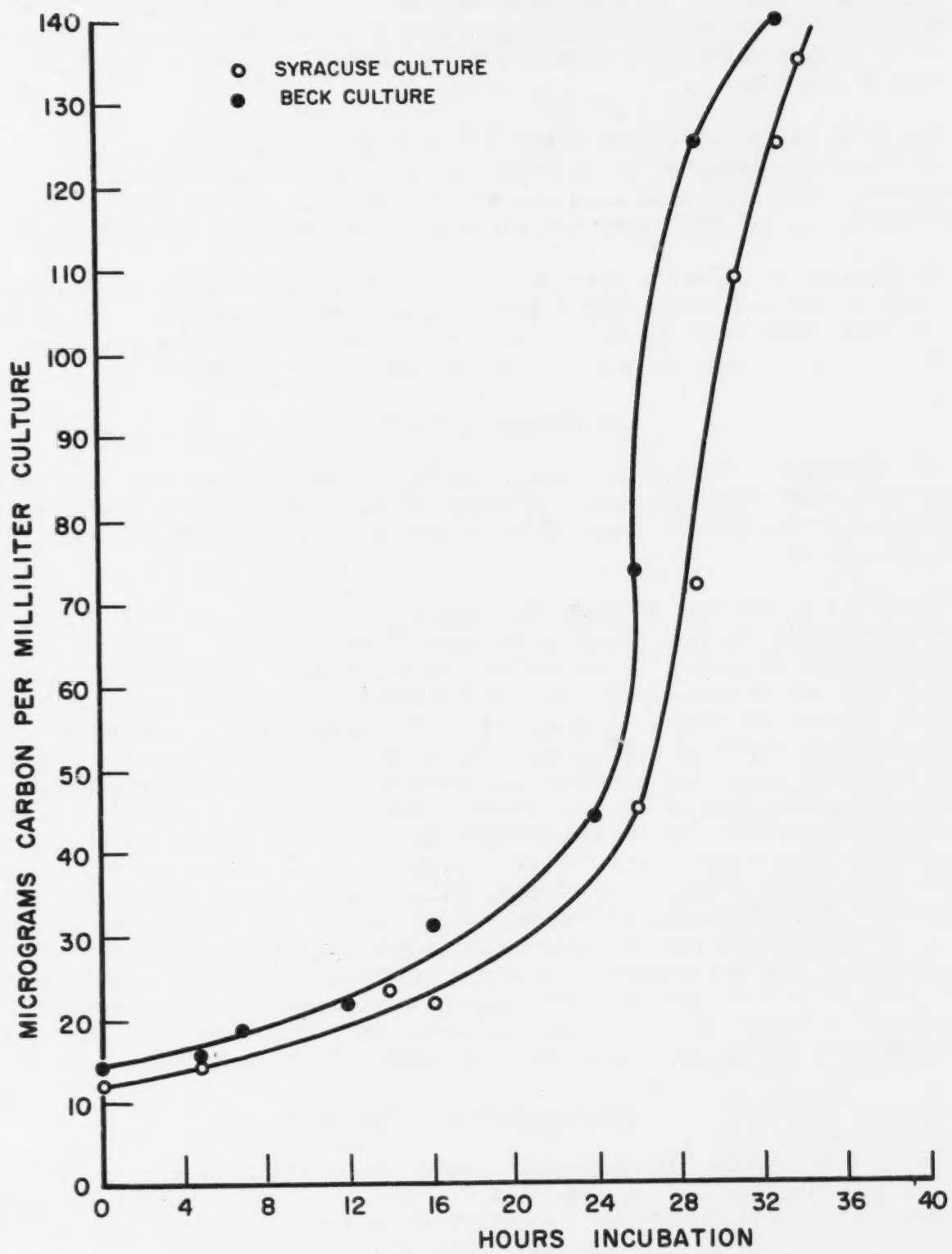


Figure 1 CARBON CONTENT OF GROWING IRON BACTERIAL CULTURES

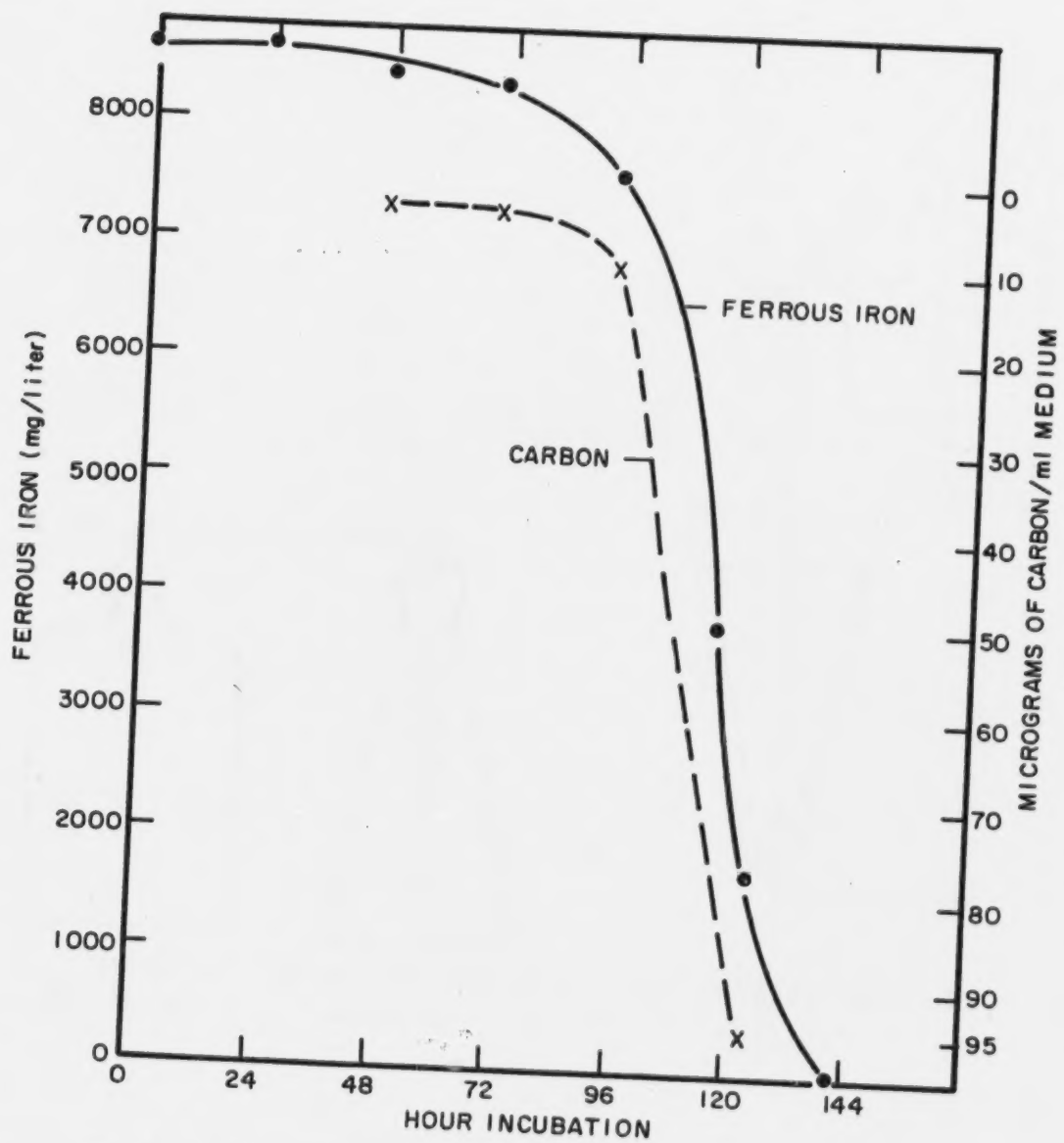


Figure 2 CARBON PRODUCTION AND FERROUS IRON OXIDATION BY SOUTHWEST-1 CULTURE

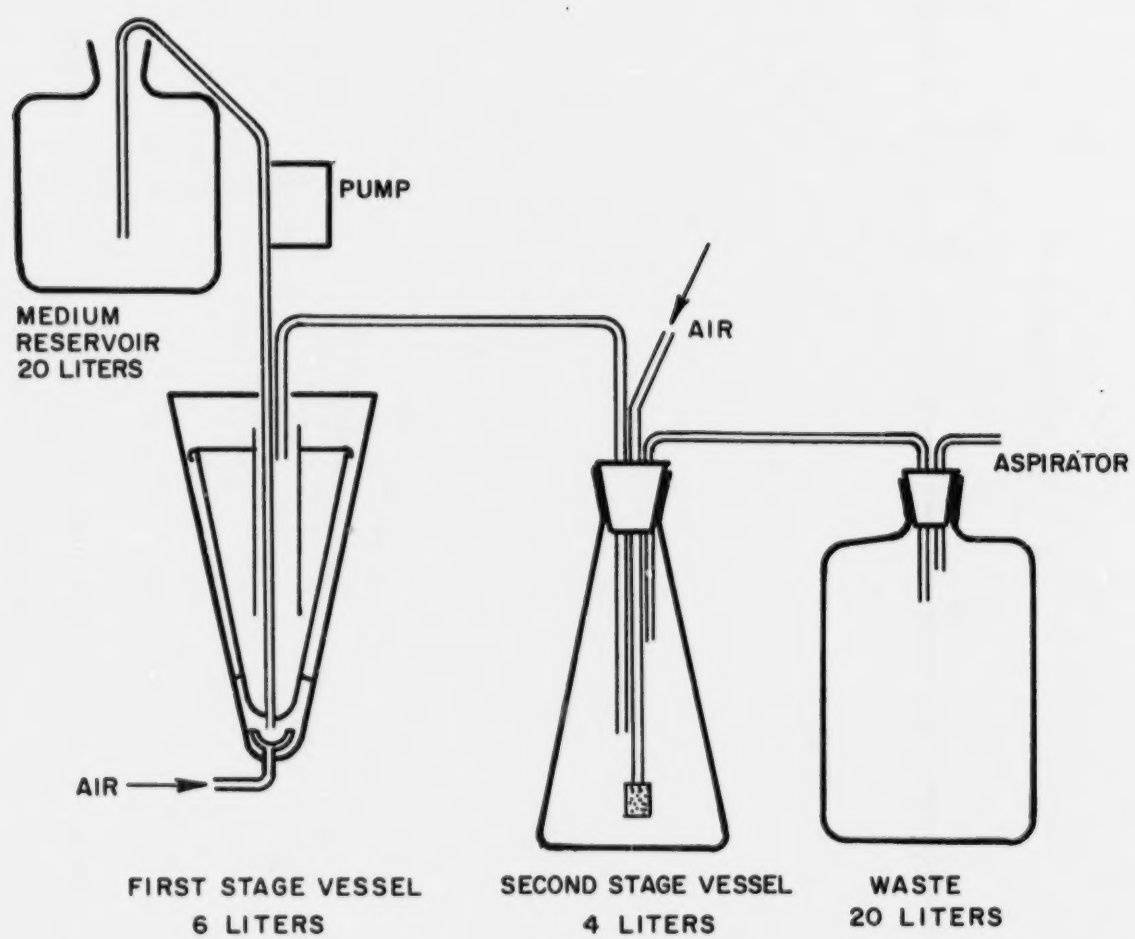


Figure 3 SCHEMATIC OF CONTINUOUS FEED BIOOXIDATION SYSTEM

water was added to a screw cap culture tube containing 10 ml of sterile API sulfate agar (Difco) at 45°C. The tube was inverted three times to distribute the sample into the medium and then allowed to solidify at room temperature. Replicate cultures of each of the waters were incubated at 35°C. After characteristic black (due to the production of iron sulfide) colonies had developed within the agar, the tubes were entered and a sterile loop was used to transfer isolates to tubes of sterile API sulfate broth containing 0.1 percent agar. After these tubes blackened, one ml of culture was withdrawn and used to inoculate a new tube of the same medium. Stock cultures were maintained in this manner until needed.

SECTION V

EXPERIMENTAL

Two-day old aliquots of three iron bacterial field cultures (Marchand, Arden and Southwest-1) and pure cultures of *Ferrobacillus* (Syracuse) and *Thiobacillus* (Beck) were used to inoculate one-liter quantities of sterile 9K medium in two-liter erlenmeyer flasks. The flasks were then incubated at 200 rpm at 25°C on a gyrotory shaker. Although all the cultures had been transferred in 9K medium at weekly intervals for six weeks, the two pure cultures displayed shorter lag periods and more rapid rates of iron oxidation than did the three field cultures (Figure 4). In all cases the cell populations reached densities of 10^7 to 10^8 bacteria per ml within 24 hours (determined by tube serial dilution). Additional oxygen supplied to the cultures by air sparging or by increased shaker speed did not detectably increase growth or iron oxidation rates. Excessive aeration was found to significantly retard rates of growth and iron oxidation.

Nutritional Requirements

In order to achieve maximal iron bacterial activity in mine discharge water, it is obviously necessary that adequate concentrations of required nutrients be present. Oxygen, carbon dioxide, nitrogen, phosphorus, ferrous iron and sulfate are nutrients that must be present in significant amounts. Chemical compositions of several acid mine drainage waters are shown in Table 3.

Nitrogen

Ammonium sulfate, sodium nitrate and sodium nitrite were quantitatively evaluated as nitrogen sources for the Southwest-1 culture.

Media containing 0, 1.0, 10 and 100 mg/l ammonium ion as the sole known source of available nitrogen were prepared by adding appropriate amounts of reagent grade $(\text{NH}_4)_2\text{SO}_4$ to modified 9K medium (modified by leaving out the amount of $(\text{NH}_4)_2\text{SO}_4$ normally present). Normal 9K medium, containing 819 mg/l ammonium ion, served as a control along with modified medium to which no ammonium salt was added. Following sterilization, the media were inoculated with equal quantities of washed Southwest-1 culture recovered from 500 ml of a 48-hour old culture grown in 9K medium. Incubation was at 25°C in 2-liter flasks on a gyrotory shaker. Resultant iron oxidation profiles are shown in Figure 5. In order to examine the possibility that available nitrogen was introduced in the test systems by inoculum carry-over in spite of the cell washings, two successive subcultures were made from these flasks into fresh sets of media and rates of iron oxidation followed. No iron oxidation or growth occurred in the subculture flask which contained no added $(\text{NH}_4)_2\text{SO}_4$ while iron oxidation and cell growth were severely limited in both subcultures containing only 1.0 mg/l ammonium ion. Assuming that the other reagent grade constituents of 9K medium did not supply available

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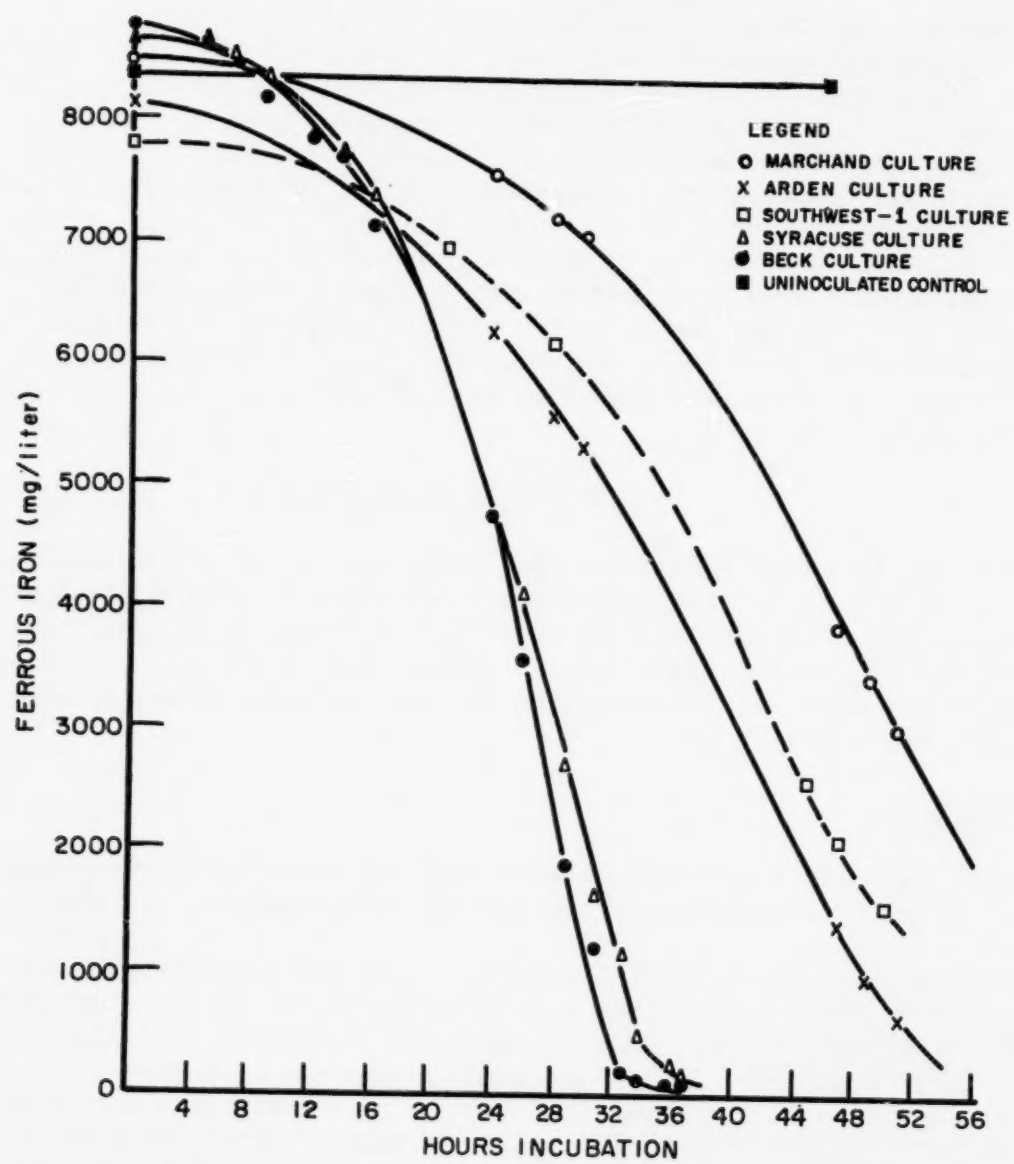


Figure 4 Fe^{++} OXIDATION RATES IN 9K MEDIUM
SHAKE FLASKS
10 % INOCULUM

TABLE 3
CHEMICAL COMPOSITIONS OF SEVERAL MINE DRAINAGE WATERS

	Mine Drainage Water - Component Concentration in mg/l			
	Core	Bolby Mill	Fetty	Arden
pH	3.1	3.6	5.2	6.2
H ₂ S	0	0	0	0
Cl	532	241	319	142
SO ₄	9400	3000	6200	1250
CO ₃	0	0	0	0
HCO ₃	0	0	34	198
Na	3360	962	2290	441
Mg	294	93	93	59
Ca	416	344	440	184
Ba	0	0	0	0
Iron (Total)	630	550	430	49
(Dissolved)	540	400	268	0
Al	99	50	0	0
Mn	11	7	4	3
P	not determined			
N (Total)	5	5	6	2
(NH ₄)	4.5	5	5	1.5
Total Salts	14600	4920	9810	2320
Suspended Solids	78	> 150	> 150	> 150
Specific Gravity	1.013	1.005	1.009	1.002
Rw-73°F Ohm Meters	0.778	2.49	1.06	3.63

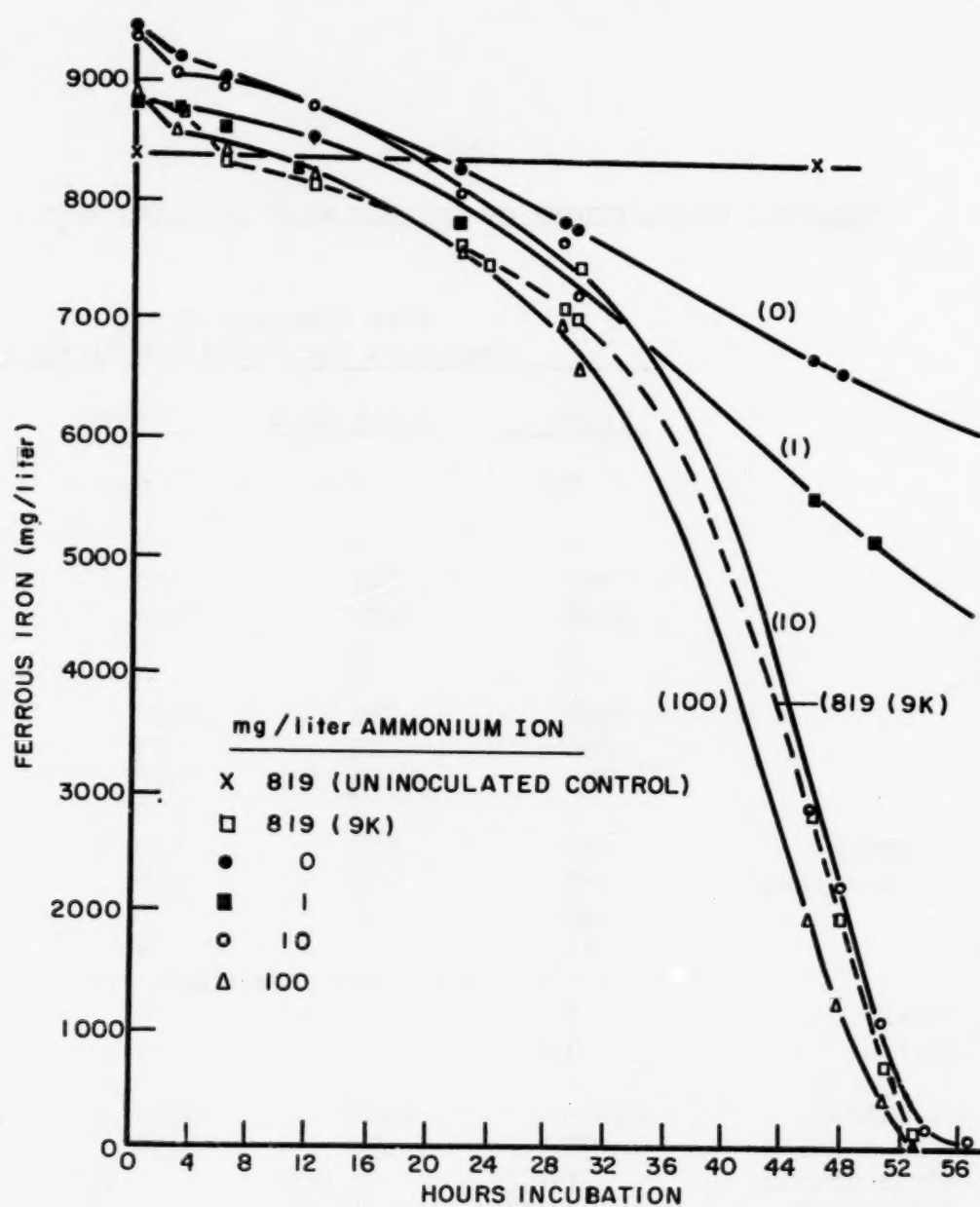


Figure 5 Fe^{++} OXIDATION AT VARIOUS AMMONIUM ION CONCENTRATIONS
SOUTHWEST-1 CULTURE -10% WASHED CELL INOCULUM

nitrogen to the organisms, it appears that one to ten mg/l of NH_4 ion is sufficient to support growth of this iron bacterial culture and accompanying ferrous iron oxidation in 9K medium.

Qualitative studies were carried out to examine the ability of the Southwest-1 culture to utilize nitrate or nitrite as a nitrogen source. Nitrate was examined at 100 and 1,000 mg/l concentrations, and nitrite was tested at 100 mg/l. When 1,000 mg/l of nitrite was added to 9K medium minus ammonium sulfate, a voluminous precipitate occurred; therefore, this concentration of nitrite was not examined. Iron oxidation at the nitrate and nitrite levels tested are shown in Figure 6. Subsequent subculture responses were identical. While these nitrogen sources can be utilized by the test organism, the ammonium salt is more readily used (compare Figures 5 and 6).

Phosphorus

Since most microorganisms utilize phosphate ion as a source of phosphorus, phosphorus requirements were studied using a phosphate salt, dibasic potassium phosphate (K_2HPO_4). Both the Southwest-1 culture and a pure culture of *T. ferrooxidans* (Beck) were used as subjects for the phosphate requirement investigation. The base medium utilized was 9K medium modified by omission of the phosphate constituent, K_2HPO_4 . K_2HPO_4 was then added to this altered 9K medium to produce media containing 0.55, 5.5 and 55 mg/l phosphate. Standard 9K medium containing 275 mg/l phosphate ion and the modified 9K base containing no added phosphate served as controls. Following inoculation with washed cells grown on 9K medium, the test flasks were incubated at room temperature on a gyrotory shaker. Subsequent iron oxidation patterns are shown in Figures 7 and 8. Results of successive subcultures carried out with the pure culture are shown in Figure 9. Although not shown in Figure 9, no ferrous iron oxidation or iron bacterial growth occurred in subcultures containing no added phosphate. Assuming no phosphate present in other medium constituents, the data reveals that phosphate becomes rate limiting for iron bacteria in 9K medium at a concentration between 0.55 and 5.5 mg/l (the Southwest-1 culture was not tested at 0.55 mg/l phosphate). Oxidation activity in the flask containing no added phosphate was apparently due to carry-over in spite of careful cell washing.

Ferrous Iron

The effect of initial ferrous iron concentration on rate of ferrous iron oxidation in batch culture was examined at concentrations of 10, 100, 500, 1,000 and 9,000 mg/l of ferrous iron. At the first four concentrations, oxidations were followed for only about six hours on the assumption that the microbial population would not significantly increase in this short period of time after inoculation. A more complete picture of the 9,000 mg/l system was obtained. Cultures used for this study were the Southwest-1, *F. ferrooxidans* (Syracuse) and *T. ferrooxidans* (Beck). Incubation was on a gyrotory shaker at 25°C. Equal volumes of 48-hour old cultures were utilized as inoculum in attempting to produce approximately equal initial

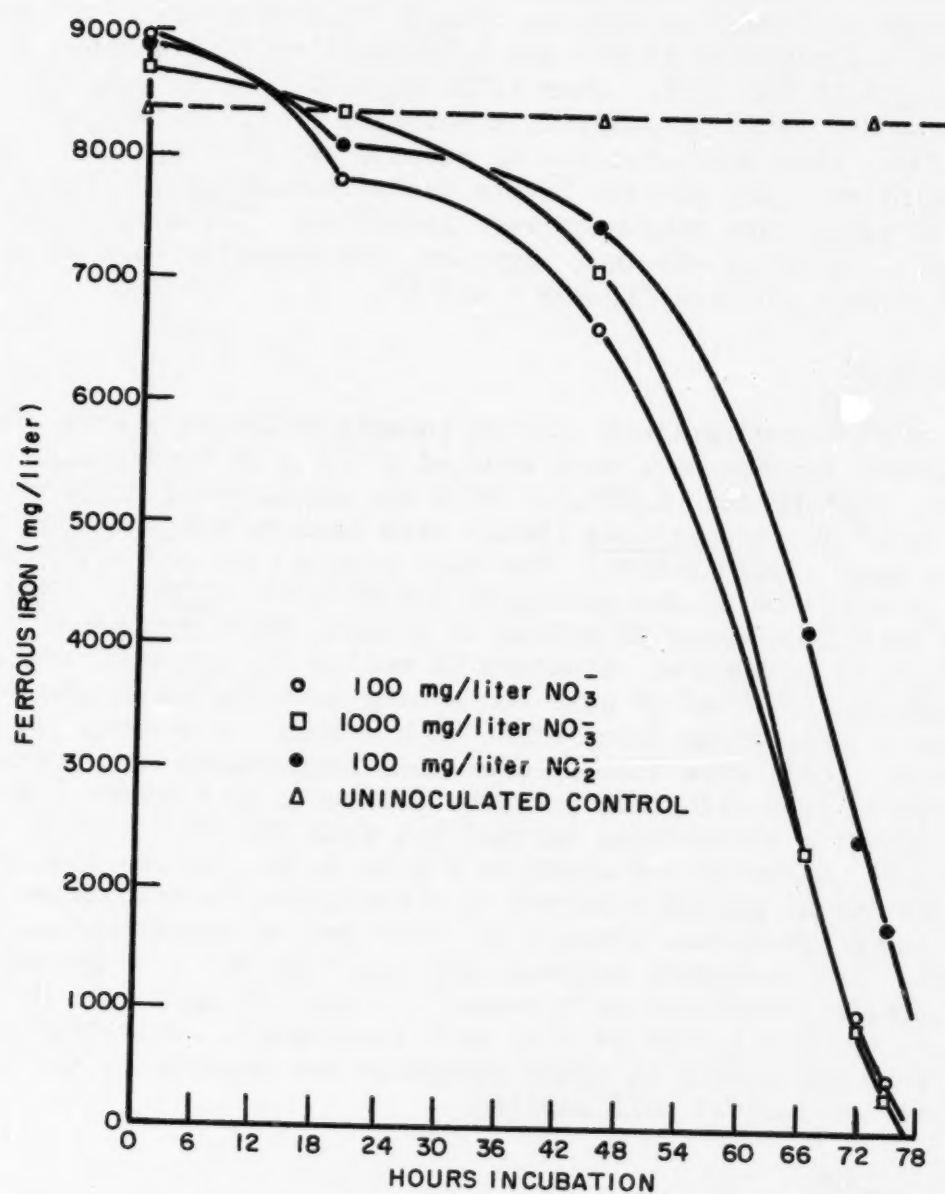


Figure 6 Fe^{II} OXIDATION USING NO_2^- OR NO_3^- AS NITROGEN SOURCE IN 9K MEDIUM SHAKE FLASKS.
SOUTHWEST-1 CULTURE - 10% WASHED CELLS INOCULUM

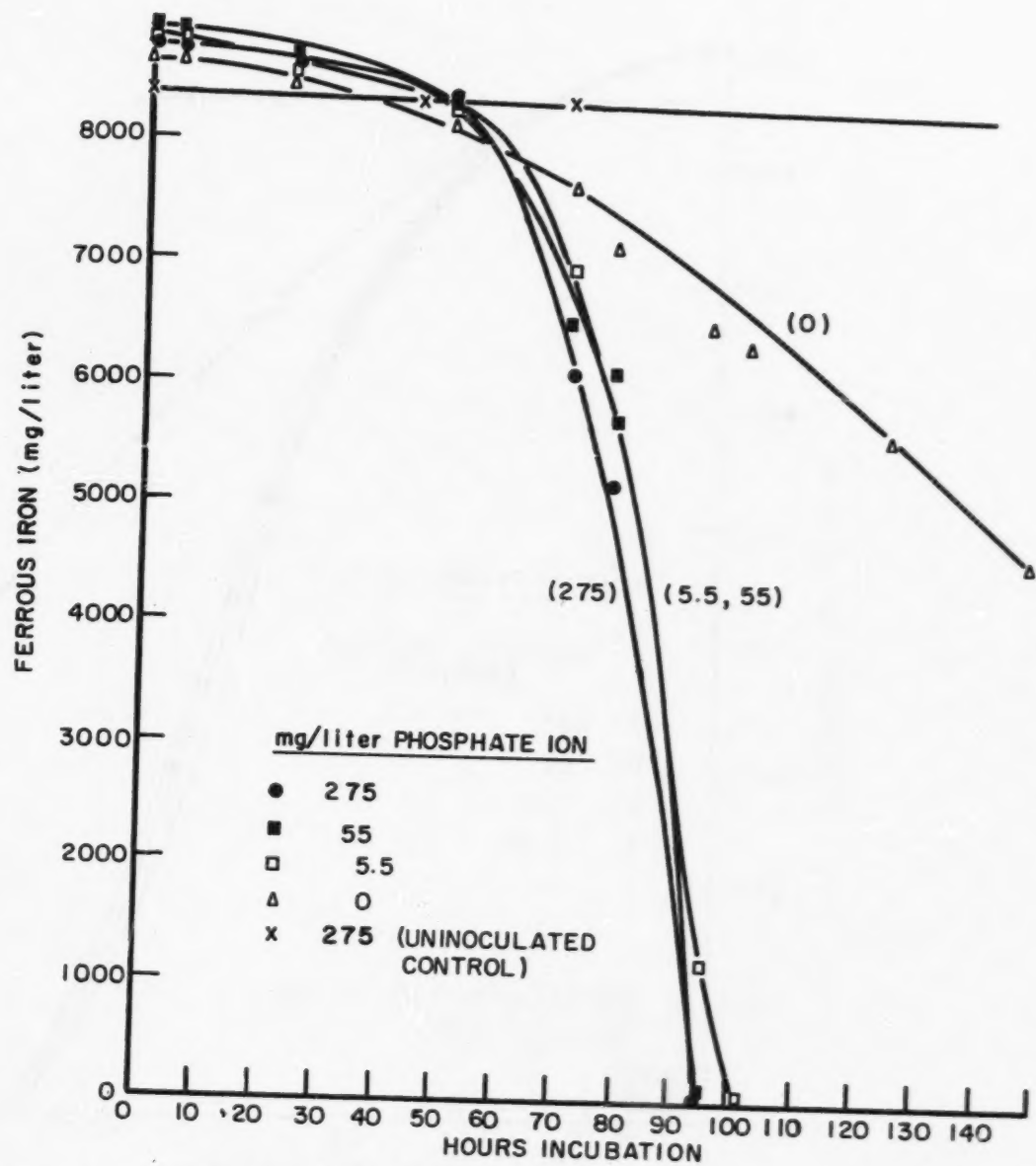


Figure 7 Fe^{+2} OXIDATION AT VARIOUS $\text{PO}_4^{=}$ CONCENTRATIONS
SOUTHWEST-1 CULTURE - 10 % WASHED CELL INOCULUM

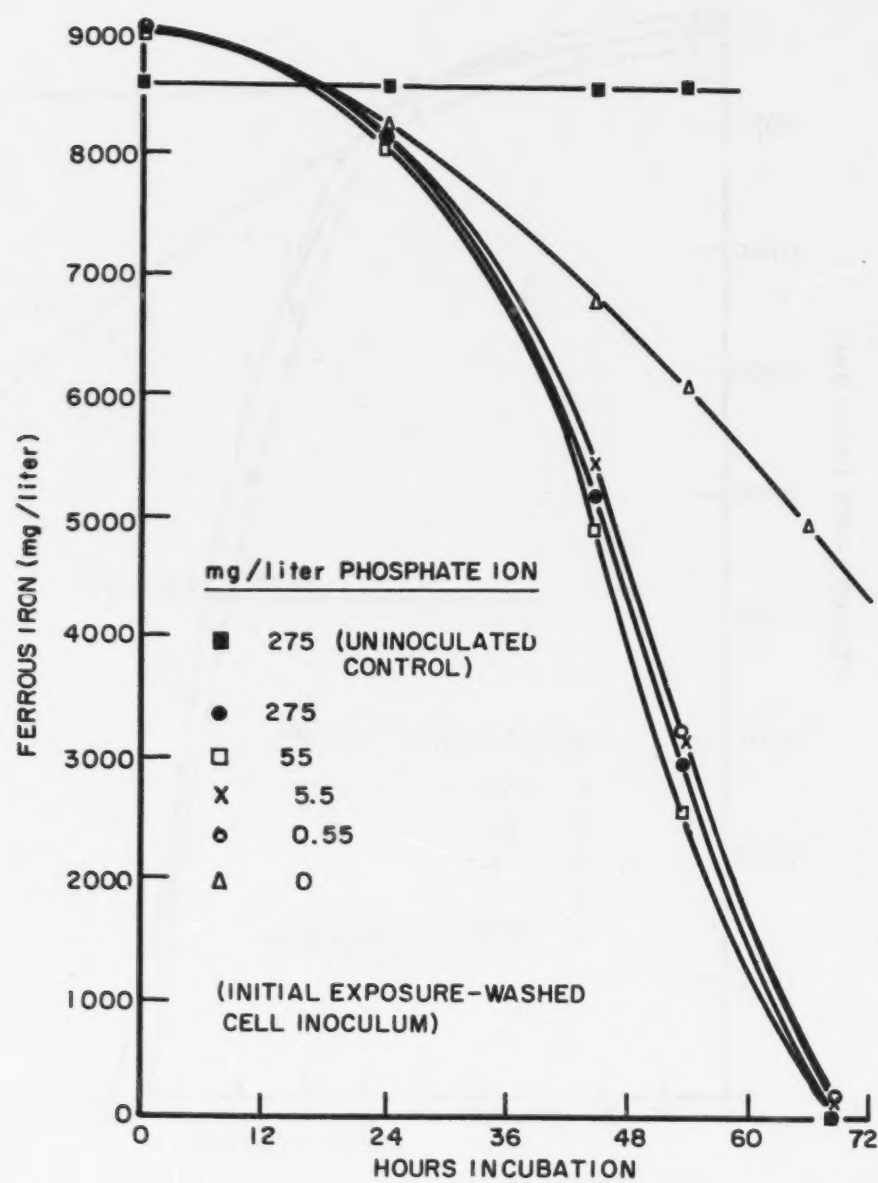


Figure 8 EFFECT OF PHOSPHATE CONCENTRATION ON FERROUS IRON OXIDATION BY THIOBACILLUS FERROOXIDANS (BECK)

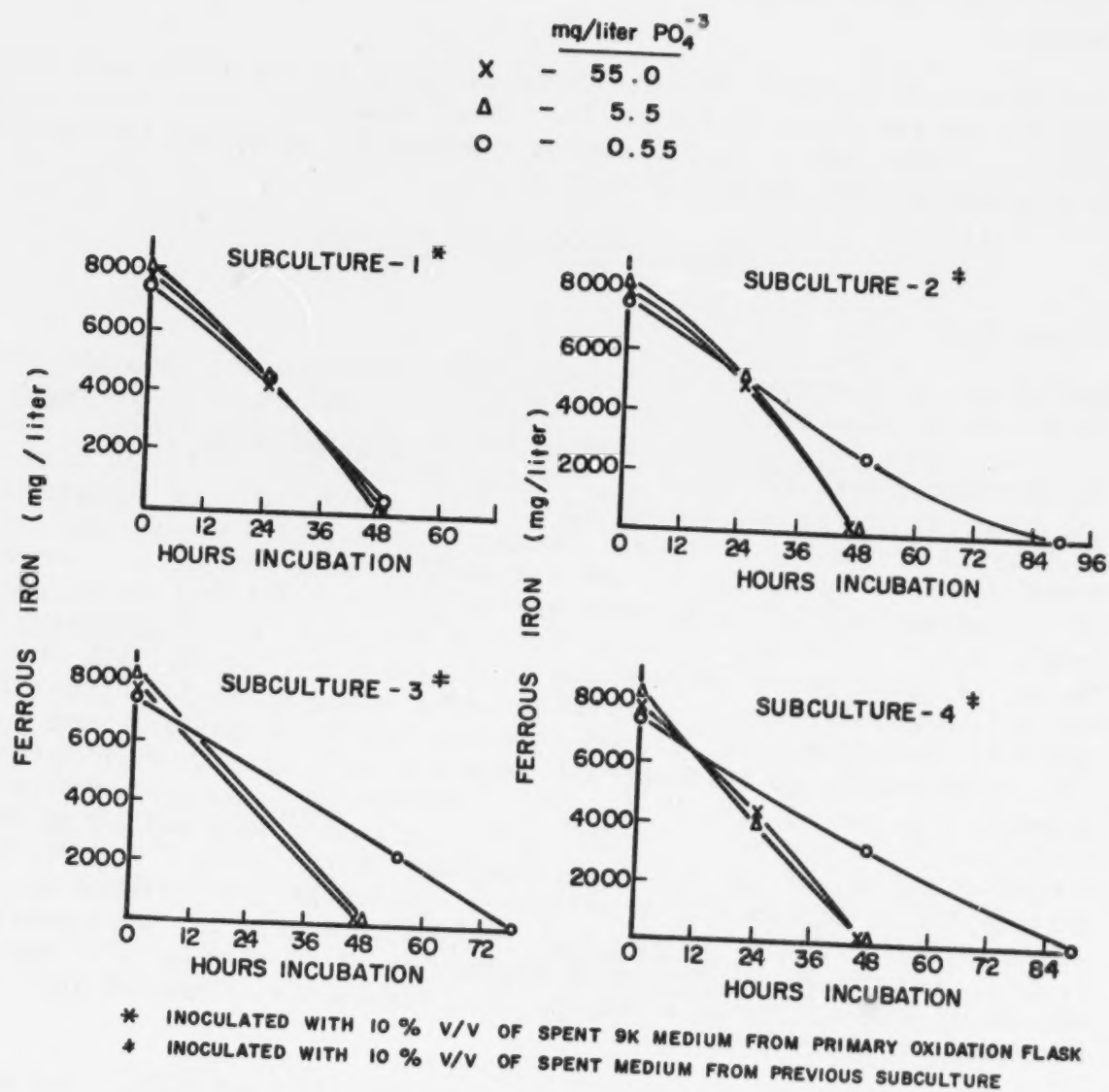


Figure 9 EFFECT OF PHOSPHATE CONCENTRATION ON FERROUS IRON OXIDATION BY T. FERROOXIDANS (BECK)-SUB-CULTURES

concentrations of cells in each flask. The oxidation of ferrous iron during the first six hours of incubation for all five concentrations of iron are shown in Figure 10 as a function of time and formation of ferric iron. Due to the similar behaviors of the three cultures under these conditions, their values were averaged to produce the functions shown in Figure 10.

Iron oxidation patterns for the three cultures in the 9,000 mg/l ferrous iron system are shown in Figure 11. These data were noted to be very similar to that for a similar system reported by Lacey and Lawson (18). This comparison is shown in Figure 12.

Other Environmental Factors

Temperature

The effect of temperature on ferrous iron oxidation was examined for four acidophilic iron bacterial cultures: Southwest-1, *F. ferrooxidans* (Syracuse), *F. ferrooxidans* (Dugan) and *T. ferrooxidans* (Beck). Temperatures studied were 10°C, 20°C, 25°C, 30°C, 35°C and 40°C. In each case the temperature was maintained at $\pm 0.5^\circ\text{C}$. Cultures were temperature acclimated by three successive transfers at each of the selected temperatures prior to inoculation. The test medium consisted of 1-liter volumes of 9K medium modified to contain approximately 1,000 mg/l of ferrous iron. The inocula were adjusted so that all test flasks contained 10^6 bacterial cells per ml at the time of inoculation. Incubation at the various temperatures was carried out using a New Brunswick PsychroTherm gyrotory shaking apparatus. Ferrous iron oxidation patterns and population developments at the selected temperatures by the four cultures are shown in Figures 13, 14, 15 and 16. Surprisingly, temperatures from 20°C to 30°C did not appear to significantly influence iron oxidation patterns exhibited by any of the cultures, and only in the case of the Southwest-1 culture did 35°C effect iron oxidation (in the latter instance oxidation was impaired). The *T. ferrooxidans* cultures (Figure 14) were unaffected by 40°C, but the *F. ferrooxidans* culture examined also at 40°C was quite adversely affected by this temperature (Figure 15). None of the cultures functioned well at 10°C.

Profiles of batch microbial ferrous iron oxidation at 10°C, 20°C and 35°C were then examined as a function of initial ferrous iron concentration. Ferrous iron levels selected for study were 10, 100, 500 and 1,000 mg/l. Results of these investigations are shown in Figures 17 through 22 and in Table 4. As may be seen, the same effect of initial ferrous iron on rate of ferrous iron oxidation that was observed at 25°C was found to hold at higher and lower temperatures. The pure culture of *T. ferrooxidans* (Beck) was more effective than the field culture at 25°C and 35°C while the field culture was slightly more effective at 10°C.

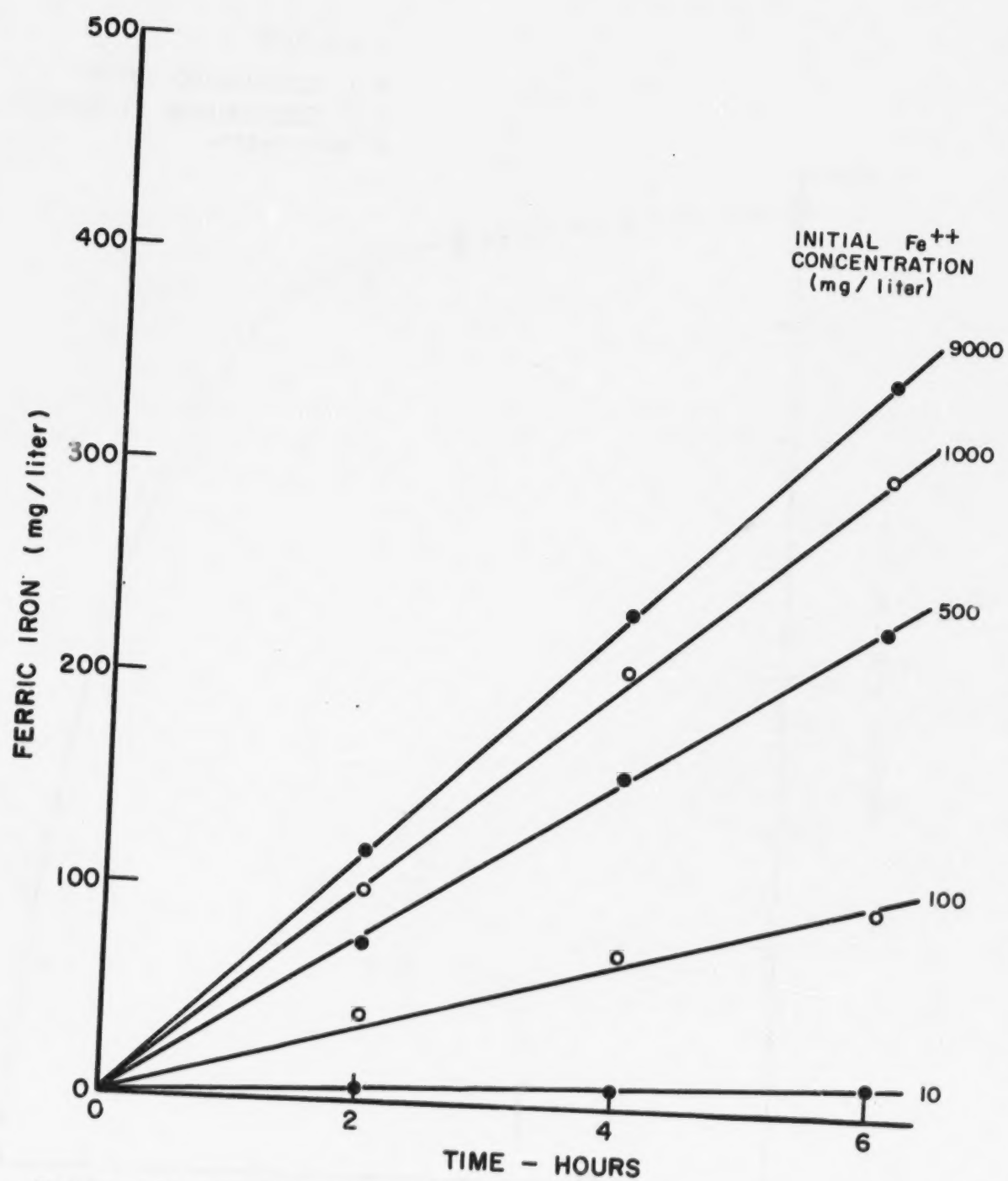


Figure 10 EFFECT OF INITIAL FERROUS IRON CONCENTRATION ON EARLY IRON OXIDATION IN BATCH CULTURE

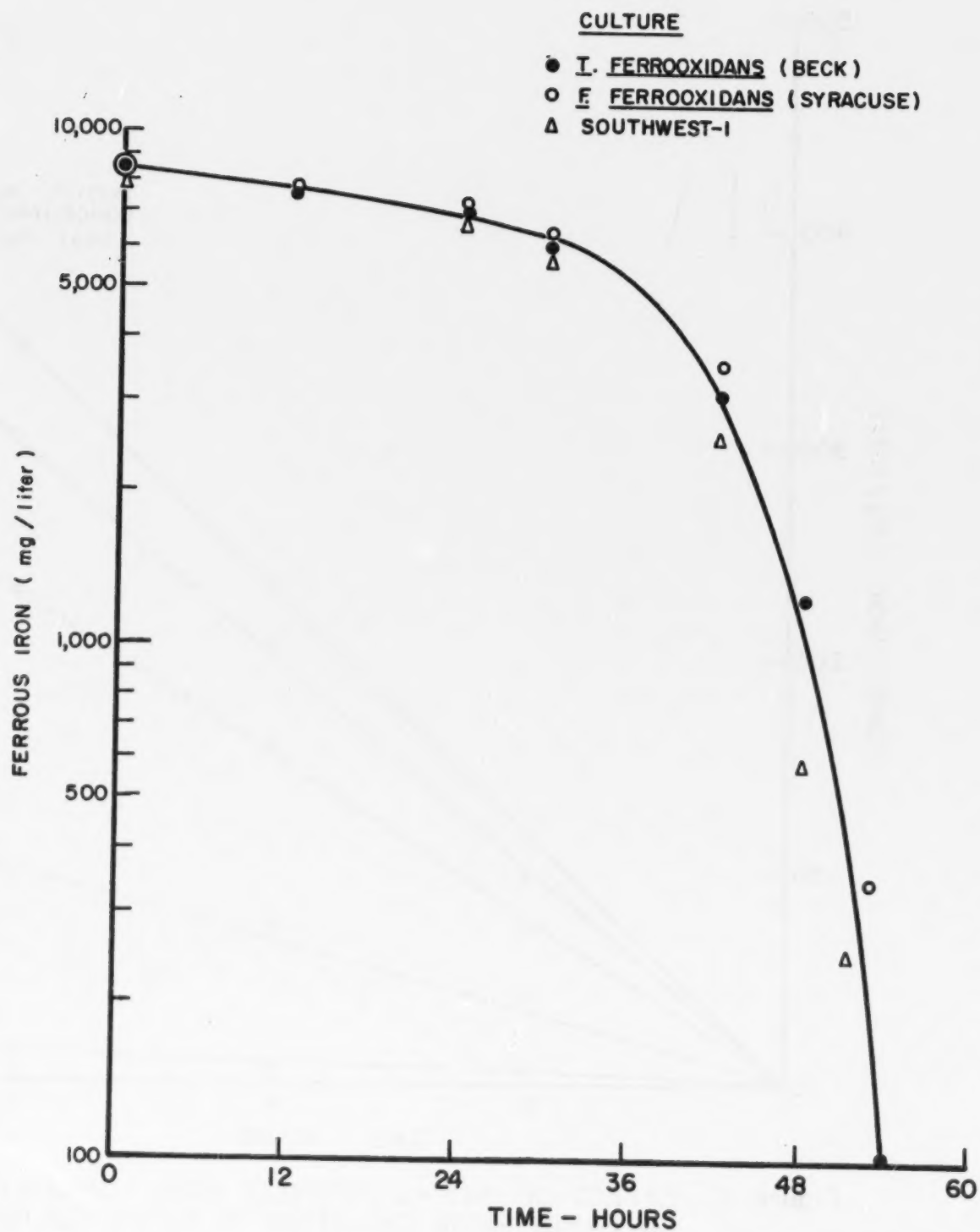


Figure II FERROUS IRON OXIDATION AT AN INITIAL IRON CONCENTRATION OF 9,000 mg/liter

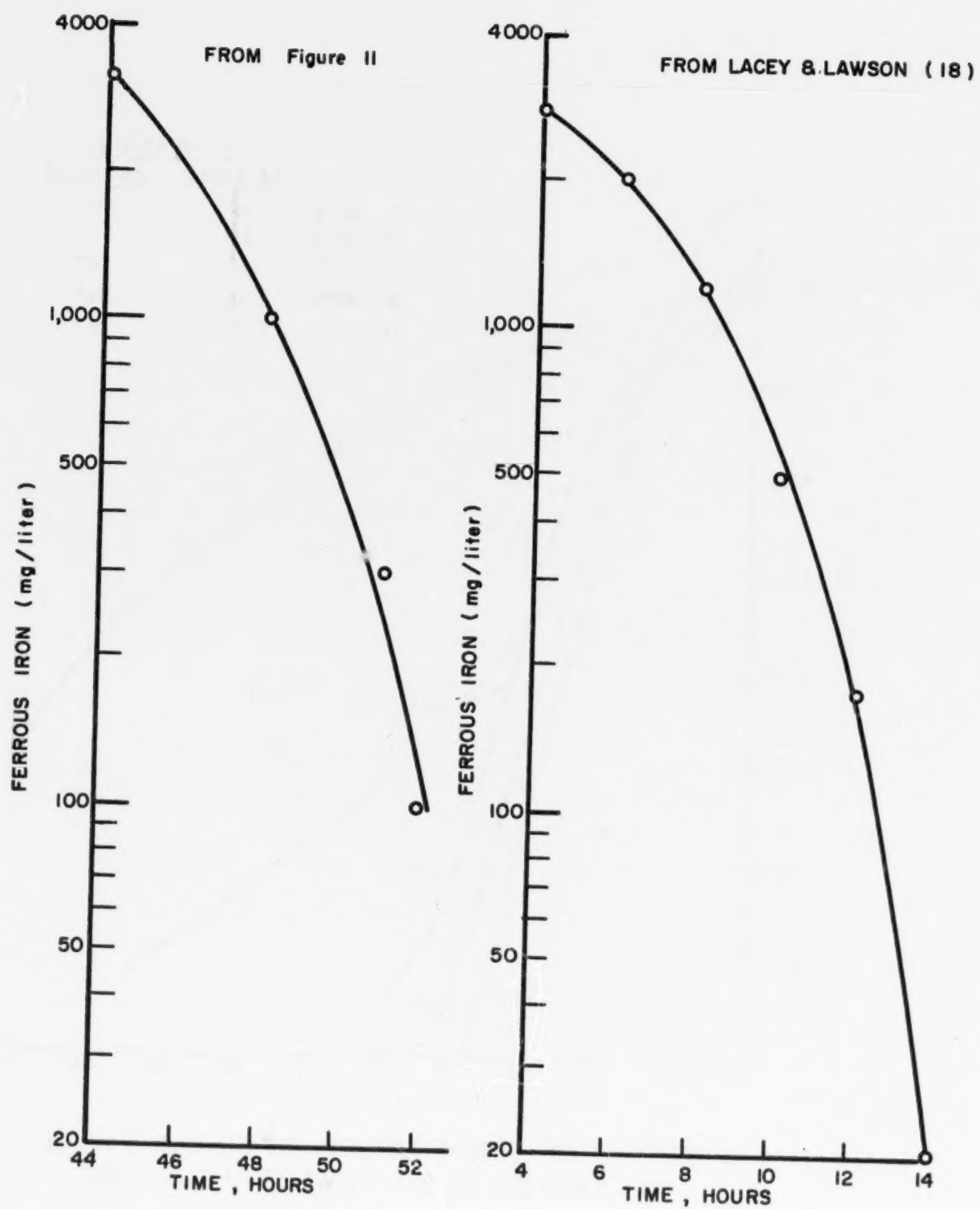


Figure 12 IRON OXIDATION AT AN INITIAL FERROUS IRON CONCENTRATION OF ABOUT 10,000 mg/liter

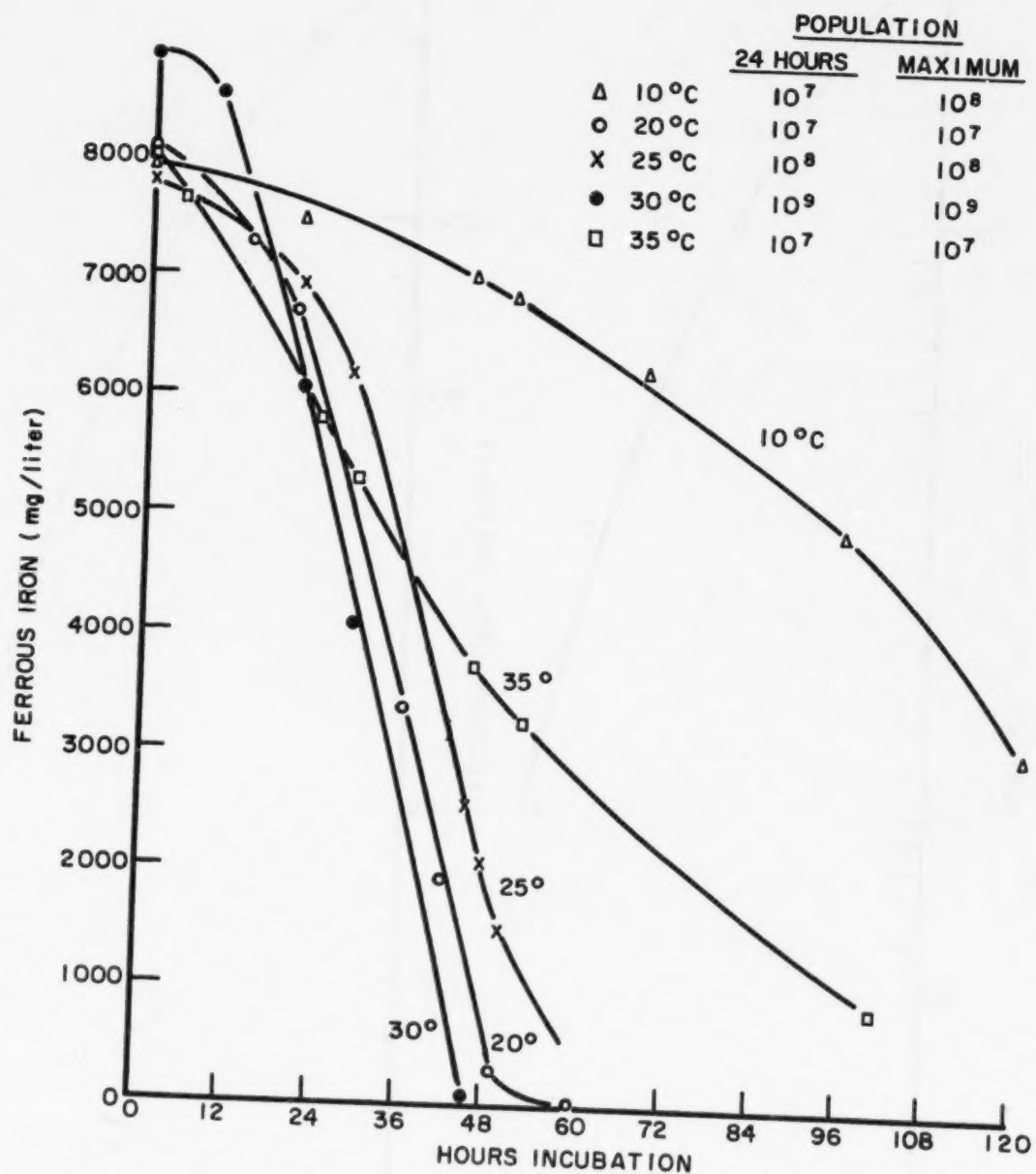


Figure 13 EFFECT OF TEMPERATURE ON FERROUS IRON OXIDATION BY SOUTHWEST-1 CULTURE

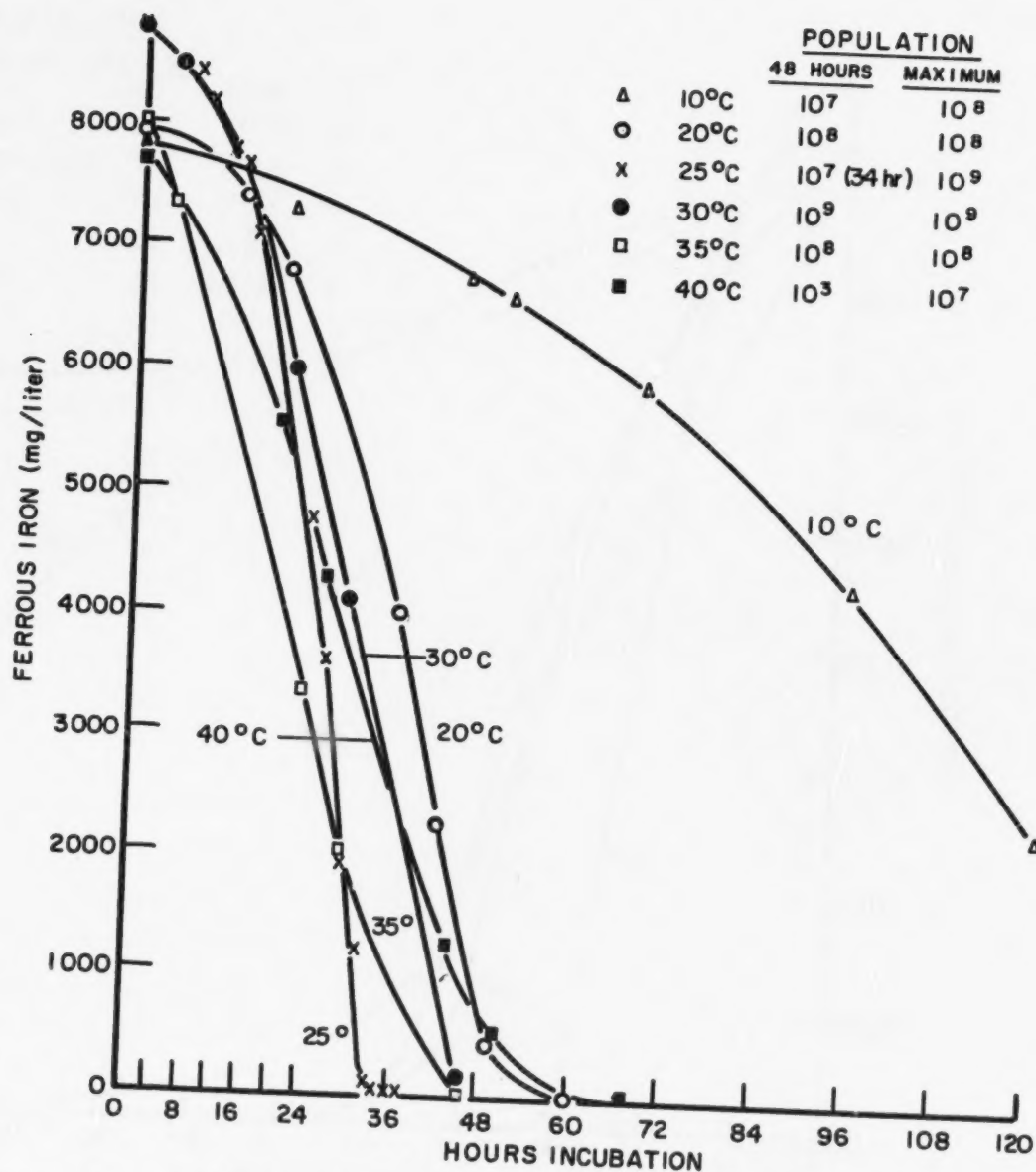


Figure 14 EFFECT OF TEMPERATURE ON FERROUS IRON OXIDATION BY THIOBACILLUS FERROOXIDANS (BECK)

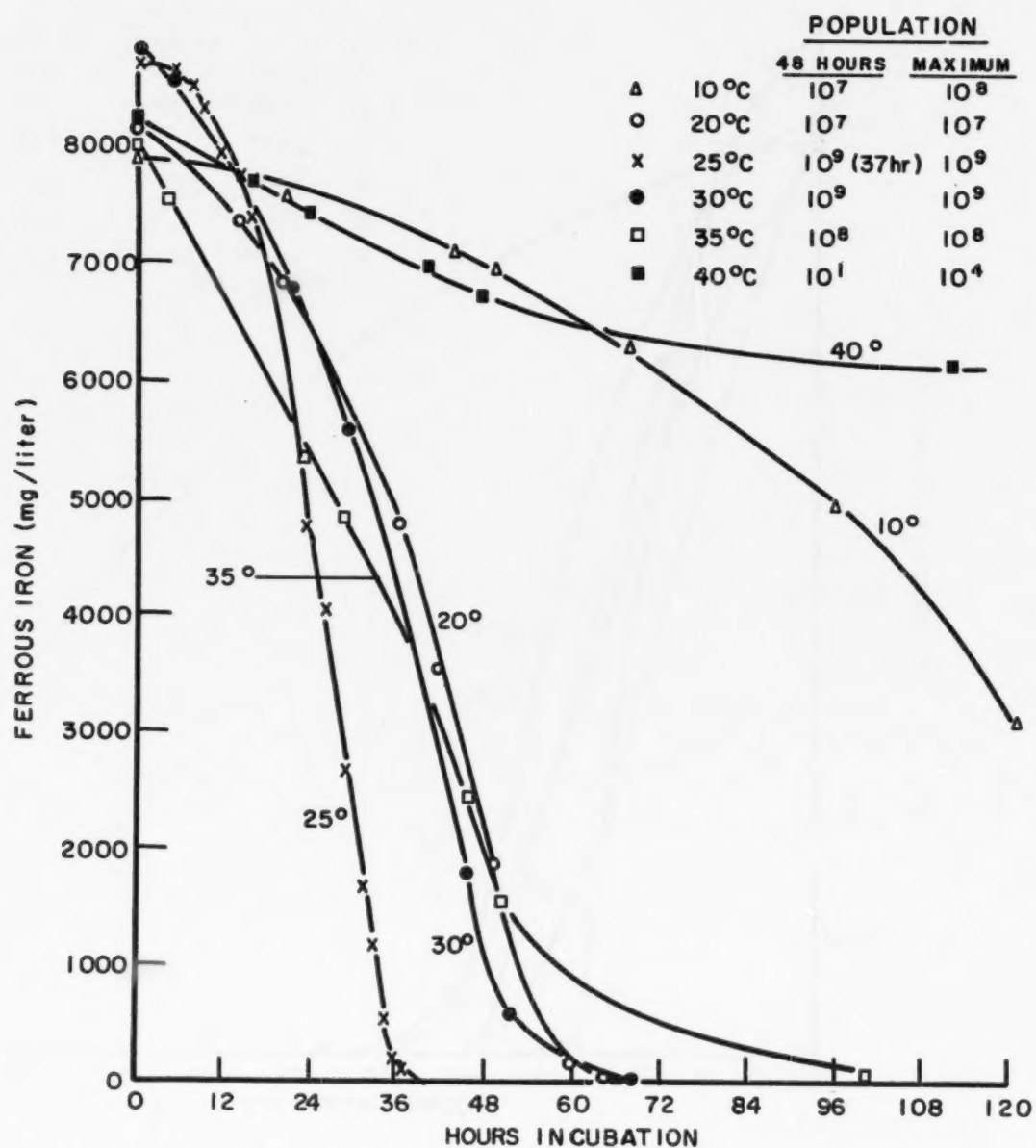


Figure 15 EFFECT OF TEMPERATURE ON FERROUS IRON OXIDATION BY FERROBACILLUS FERROOXIDANS (SYRACUSE)

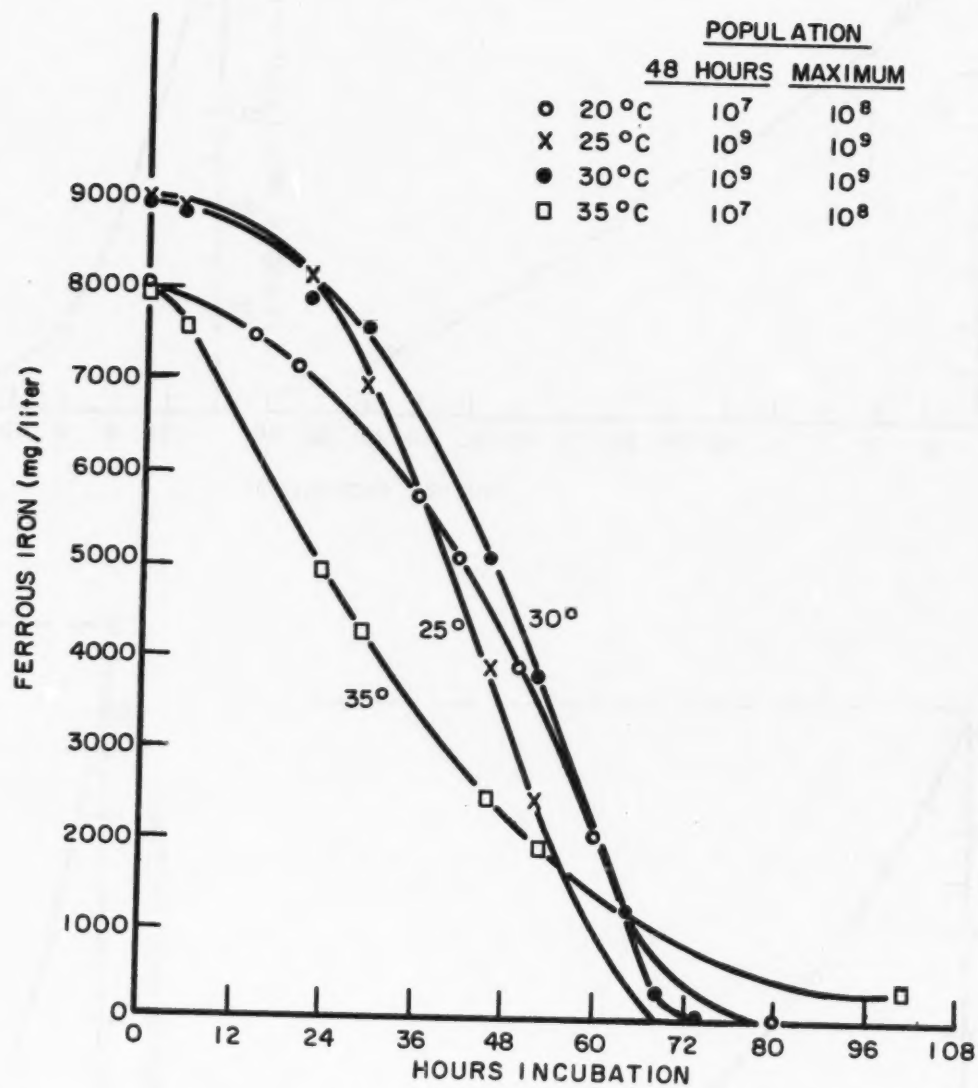


Figure 16 EFFECT OF TEMPERATURE ON FERROUS IRON OXIDATION BY FERROBACILLUS FERROOXIDANS (DUGAN)

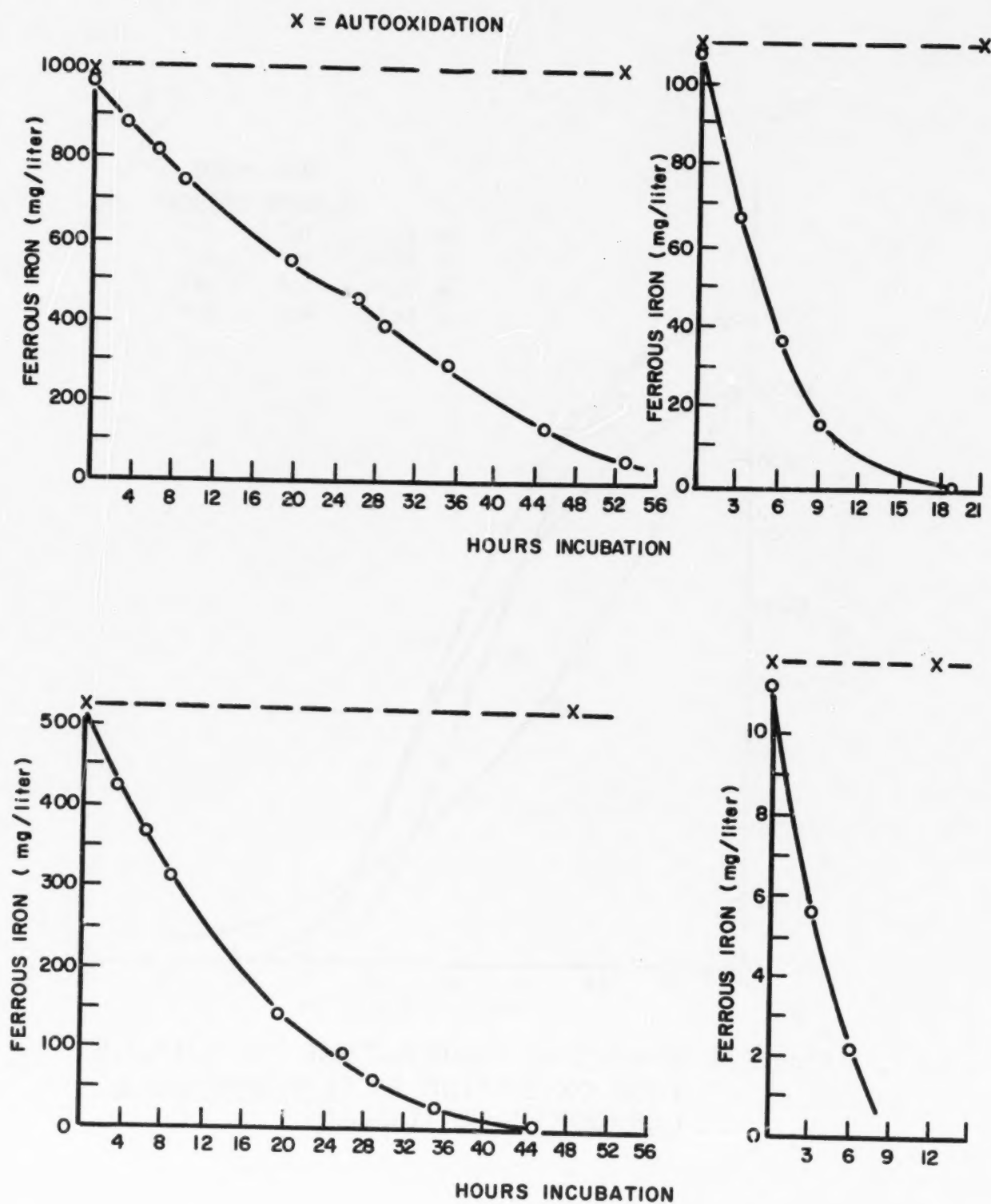


Figure 17 FERROUS IRON OXIDATION AT VARIED INITIAL Fe^{++} CONCENTRATIONS BY BECK CULTURE-SHAKE FLASKS 10°C

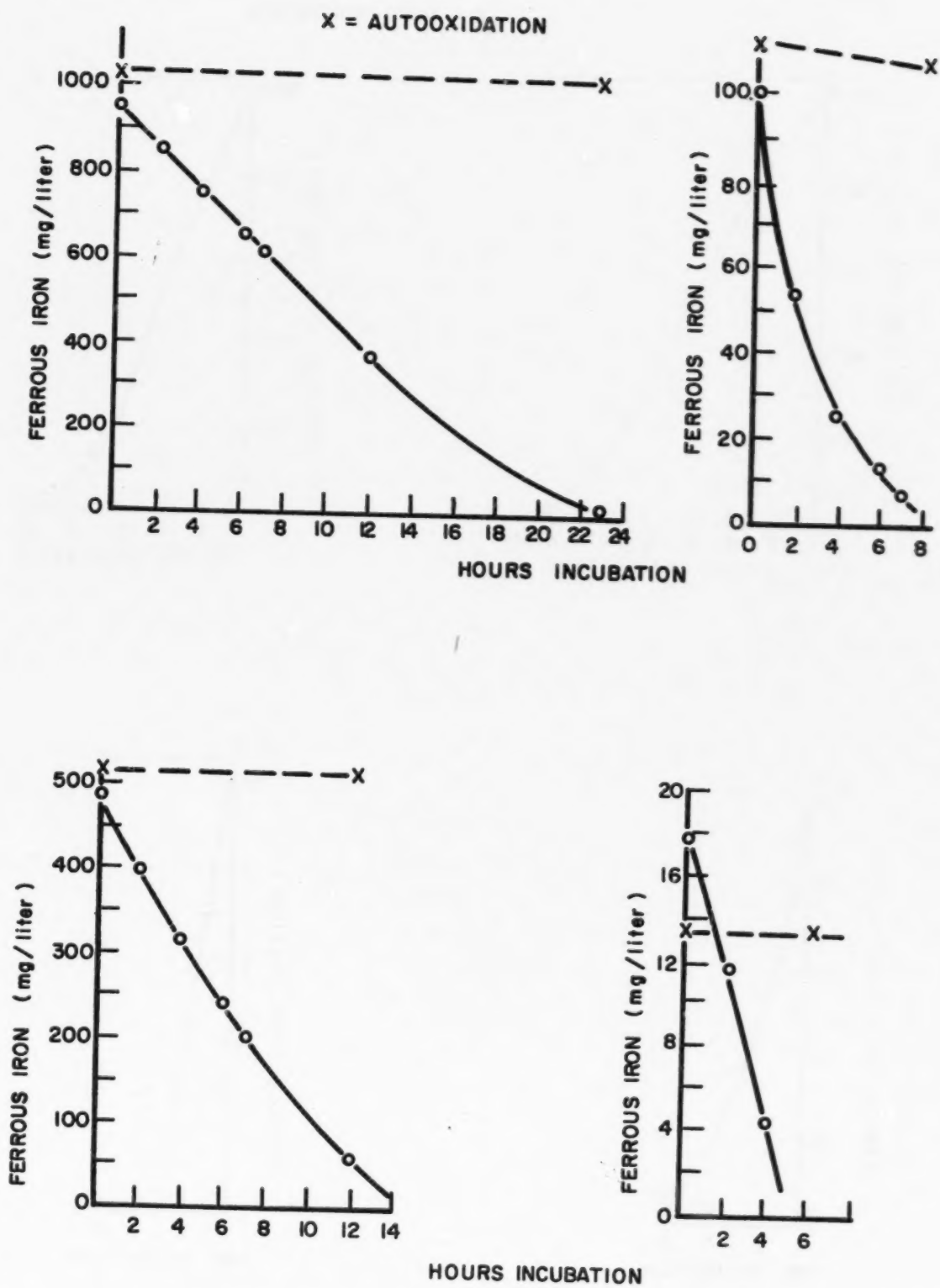


Figure 18 FERROUS IRON OXIDATION AT VARIED INITIAL Fe^{++} CONCENTRATIONS BY BECK CULTURE-SHAKE FLASKS 20°C

X = AUTO-OXIDATION

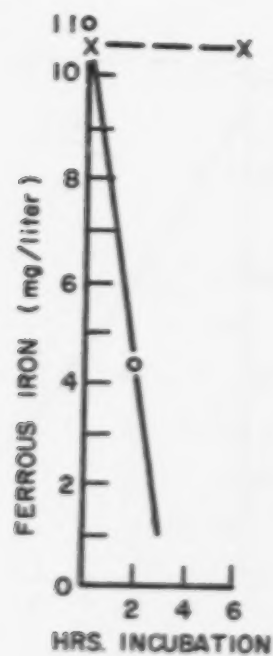
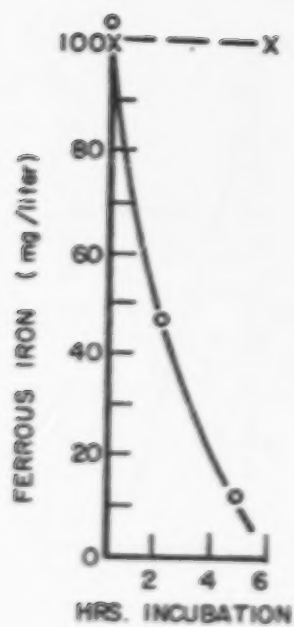
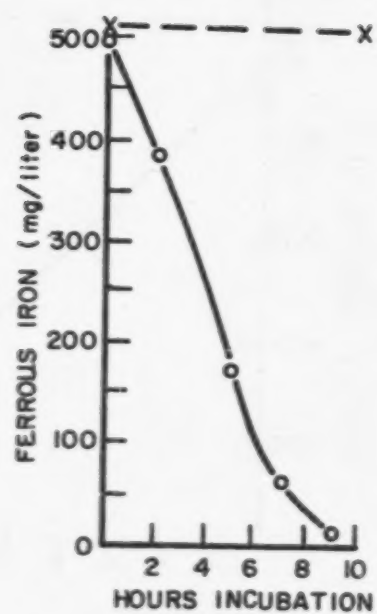
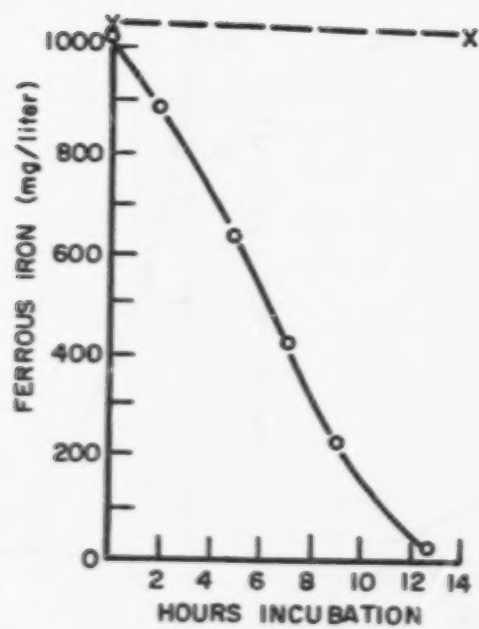


Figure 19 FERROUS IRON OXIDATION AT VARIED INITIAL Fe^{++} CONCENTRATIONS BY BECK CULTURE-SHAKE FLASKS 35°C .

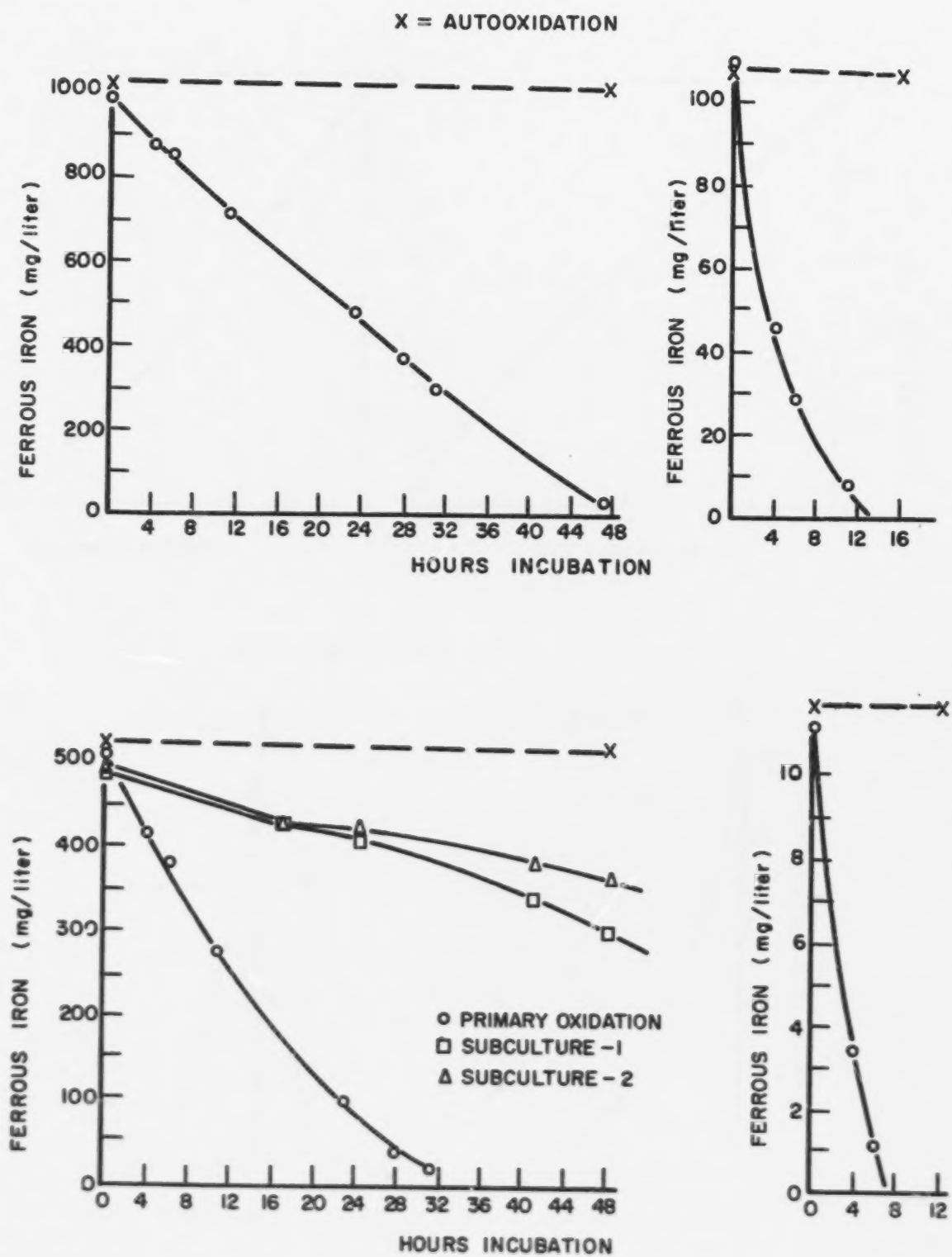
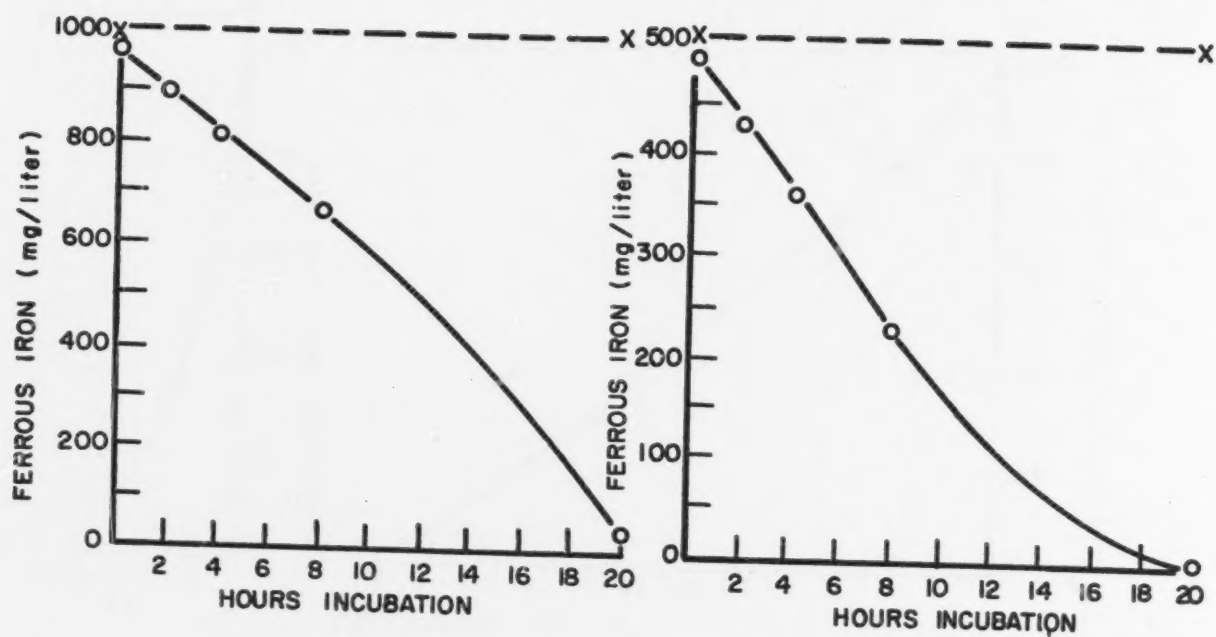


Figure 20 FERROUS IRON OXIDATION AT VARIED INITIAL Fe^{++} CONCENTRATIONS BY SOUTHWEST-1 CULTURE - SHAKE FLASKS 10°C



X = AUTO-OXIDATION

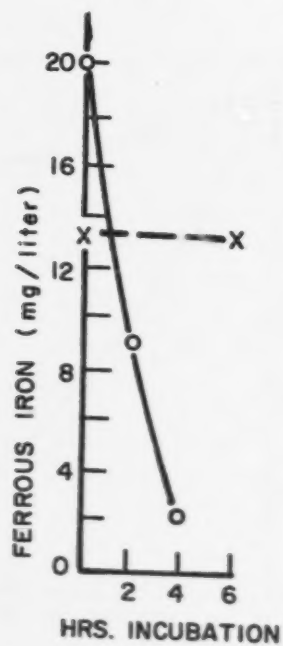
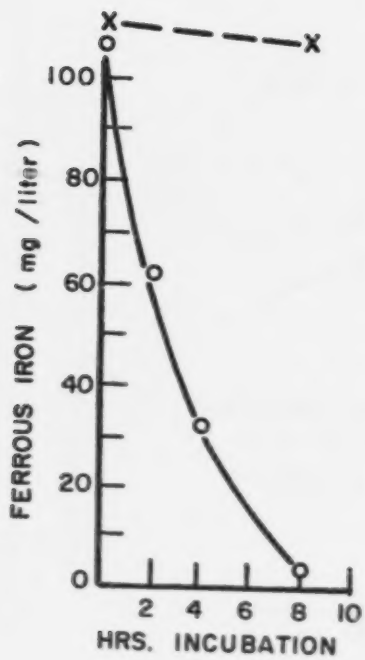
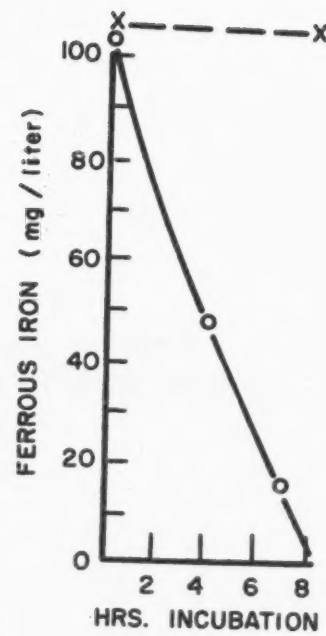
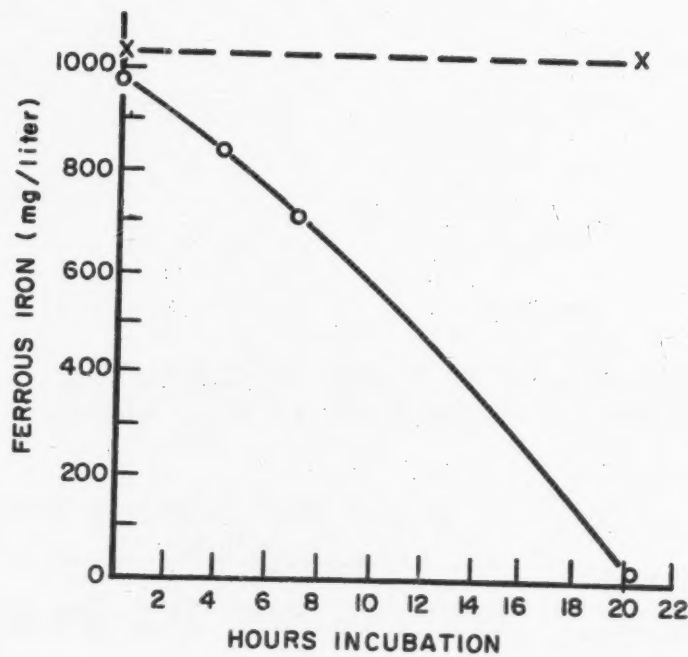


Figure 21 FERROUS IRON OXIDATION AT VARIED INITIAL Fe^{++} CONCENTRATIONS BY SOUTHWEST-1 CULTURE - SHAKE FLASKS 20°C



X = AUTO-OXIDATION

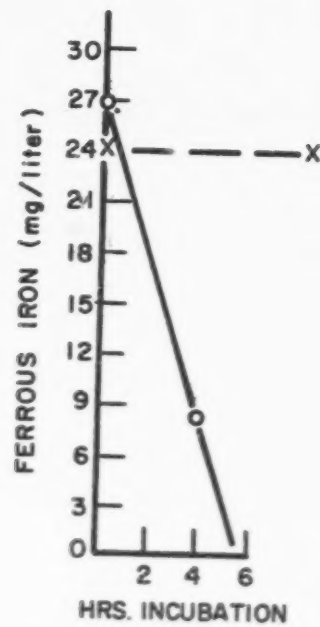
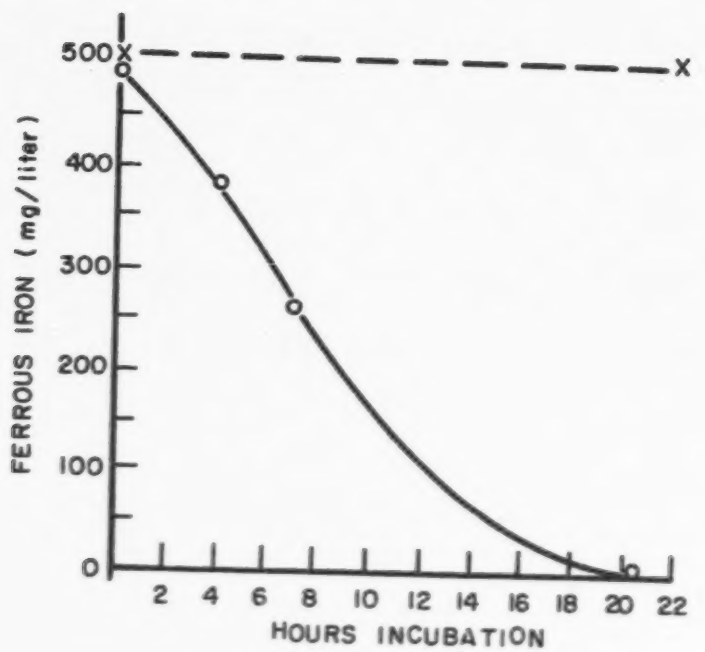


Figure 22 FERROUS IRON OXIDATION AT VARIED INITIAL Fe^{++} CONCENTRATIONS BY SOUTHWEST-1 CULTURE - SHAKE FLASKS 35 °C.

TABLE 4

FERROUS IRON OXIDATION RATES AT VARIED INITIAL Fe^{++}
CONCENTRATIONS AND INCUBATION TEMPERATURES IN SHAKE FLASKS

	Initial mg/l Fe^{++}	Average Fe^{++} Oxidation Rate mg/l/hr				
		10°C	20°C	25°C	35°C	Controls
Beck Culture	10	1.5	3.4	2.2	3.4	0
	100	9.3	13.4	13.0	18.8	0
	500	11.0	35.5	32.7	53.3	0
	1000	17.0	40.5	40.4	80.4	0
Southwest-1 Culture	10	1.7	4.5	1.3	4.7	0
	100	9.3	12.8	8.0	12.4	0
	500	16.0	24.8	32.7	24.0	0
	1000	20.6	46.1	40.4	47.2	0

Initial pH

It has been established in the literature that acidophilic iron bacteria grow and oxidize ferrous iron poorly at pH values above 4 (19). However, air oxidation of ferrous iron occurs more rapidly as the pH is raised from 4, and acidity is produced when ferrous iron is oxidized by air or by microbial catalysis. Thus, if some oxidation of ferrous iron takes place, regardless of the mechanism, the pH of the solution decreases unless highly buffered. In view of these factors, it seemed reasonable to assume that microbial oxidation of ferrous iron might take place reasonably well even in systems having an initial or influent pH of 4 or above.

Using three different iron bacterial cultures, Southwest-1, T. ferrooxidans (Beck) and F. ferrooxidans (Syracuse), the effect of initial pH on microbial oxidation of ferrous iron was examined. The studies were carried out batchwise in shake flasks at 25°C using 9K medium modified to contain about 500 mg/l ferrous iron. The cultures were used to inoculate sets of flasks in which the medium had been adjusted to pH 3, 4, 5 or 6 with potassium hydroxide. Iron oxidation rates were monitored. Uninoculated flasks at the four different pH values were used to determine the extent of auto-oxidation under these conditions as well as accompanying pH changes. Results are presented in Table 5 and in Figure 23.

As may be seen, no ferrous iron oxidation or pH shift took place at pH 3 in the absence of microbial growth. Rapid ferrous iron oxidation with no lag period was displayed at pH 3 by all three cultures. There was a small amount of ferrous iron oxidation by air at pH 4 with a concomitant decrease in pH of about 0.5 unit. The T. ferrooxidans culture appeared to be inhibited until this pH shift occurred and then displayed a rather long lag period before rapidly oxidizing the ferrous iron present. The other two cultures appeared to be essentially unaffected by pH 4.

At pH 5 there was a significant degree of ferrous iron oxidation by air, and it occurred in the first few hours of incubation. While all three cultures appeared to be inactive until the pH was dropped by air oxidation of the iron, only the T. ferrooxidans culture displayed a relatively long lag period once the pH had dropped.

As might be expected, more air oxidation of iron was found at pH 6; and a more pronounced effect on microbial oxidation of iron was noted at this pH. All of the cultures displayed a significant lag period at this pH with again the Thiobacillus culture being effected most severely.

Polyelectrolyte Catalyst

The polyelectrolyte, sodium polyvinylsulfonate, has been reported to be a very effective catalyst for some oxidation reactions at a concentration of 1 gram of polyelectrolyte per liter (20). When this polymer was added

TABLE 5

AIR OXIDATION OF FERROUS IRON AT SEVERAL
INITIAL pH VALUES AT 25°C

Initial pH adjusted with KOH, uninoculated flasks
incubated on a gyrotory shaker at 200 rpm

	Hours Incubated			
	0	4	24	48
Ferrous Iron*	429	425	425	425
pH	3.0	3.0	3.0	3.0
Ferrous Iron*	429	420	420	422
pH	4.0	3.8	3.6	3.5
Ferrous Iron*	429	385	384	384
pH	5.0	3.7	3.7	3.7
Ferrous Iron*	429	340	337	330
pH	6.0	4.2	4.1	2.8

* mg Fe⁺⁺/l

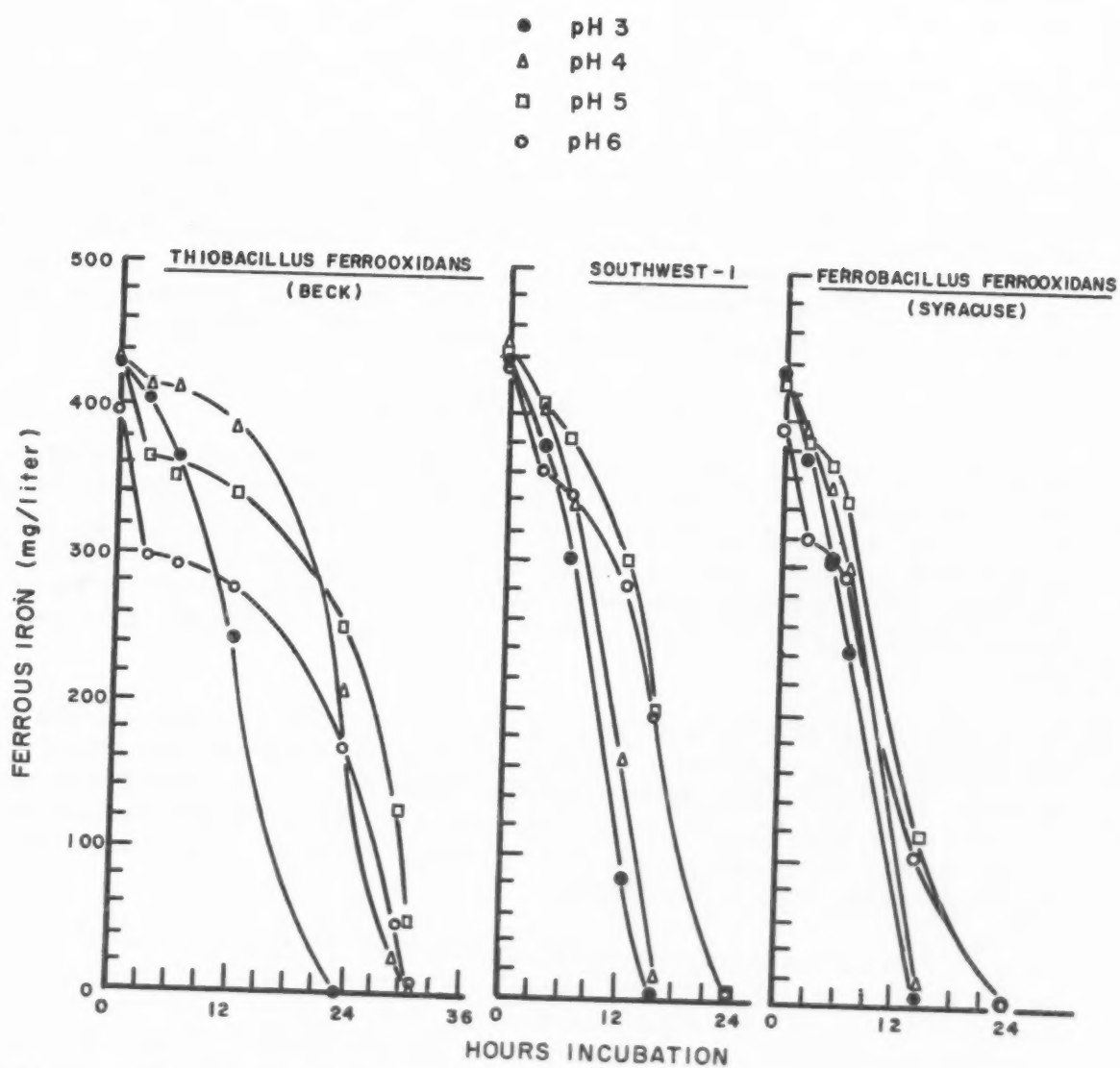


Figure 23 EFFECT OF INITIAL pH ON FERROUS OXIDATION IN SHAKE FLASKS - 25°C

to 9K medium containing 500 mg/l of ferrous iron and inoculated with Southwest-1 culture, a poorer rate of iron oxidation was experienced than in a control system void of the polymer (see Table 6). Subsequent investigation showed that the polymer also inhibited air oxidation of ferrous iron.

Redwood Leachate

It became important to consider potential effects of water soluble components of redwood on iron bacterial oxidation of ferrous iron when tanks constructed of redwood were being considered for pilot plant oxidation vessels. Redwood shavings were added to 9K medium which was then inoculated with the Southwest-1 iron bacterial culture. Resultant ferrous iron oxidation data (Figure 24) indicated that the presence of redwood did not significantly effect the iron oxidation pattern.

Continuous Culture Studies

With few exceptions waste treatment is most economically accomplished using continuous flow systems. While batch data is useful in suggesting continuous culture conditions, it is usually wise to actually carry out continuous culture studies prior to the design and construction of larger systems. Therefore, a small bench-scale continuous feed system was assembled and used to check out factors suggested by the previous batch work.

To ascertain the effect of air oxidation of ferrous iron in the system, 9K medium was fed at rates to give 87- or 12-hour average residence periods in the oxidation reservoir. Each residence period was examined for two-week periods of operation, and 1/3 VVM of aeration was utilized. No microbial inoculation was made, and no ferrous iron oxidation was observed under either condition.

To establish an active microbial population in the system, the first oxidation vessel was filled to the six-liter mark with nonsterile 9K medium and inoculated with the Southwest-1 culture. The medium was aerated at 1/3 VVM and incubated at room temperature (controlled at 25°C). After iron oxidation began to occur, ferrous iron (Fe_2SO_4) was added on a batch basis until oxidation was found to be occurring at a rapid rate. Continuous feed of 9K medium and withdrawal was then initiated at rates calculated to give an average residence time of 87 hours in the oxidation vessel. Steady-state operation was maintained for 7 days, and then the flow rate was increased to give an average residence time of 77 hours. Following this pattern of establishing steady state then increasing flow rate, it was found that the residence time could be decreased to 14 hours without upset. At a 12-hour residence period, steady-state operation could not be maintained. Data from these studies are shown in Table 7.

From the results shown in Table 7, it may be seen that, at a feed of 9,000 mg/l ferrous iron, the minimum residence time that can be maintained

TABLE 6
EFFECT OF SODIUM POLYVINYLSULFONATE ON THE
BIO-OXIDATION OF FERROUS IRON IN SHAKE FLASKS

<u>Hours Incubation</u>	<u>Residual Ferrous Iron (mg/l)</u>		
	<u>Inoculated Medium*</u>	<u>Inoculated Medium + SPVS**</u>	<u>Uninoculated Medium + SPVS**</u>
0	510	478.5	458.4
16	478.5	431.5	458.4
24.75	409.2	427.1	
40.75	33.5	424.8	458.4
64.25	0	380.1	458.4
90	0	58.1	458.4

* Medium = 9K medium containing 500 mg/l ferrous iron

** SPVS = sodium polyvinylsulfonate - Hoechst Chemical Company
molecular weight $\approx$ 20,000

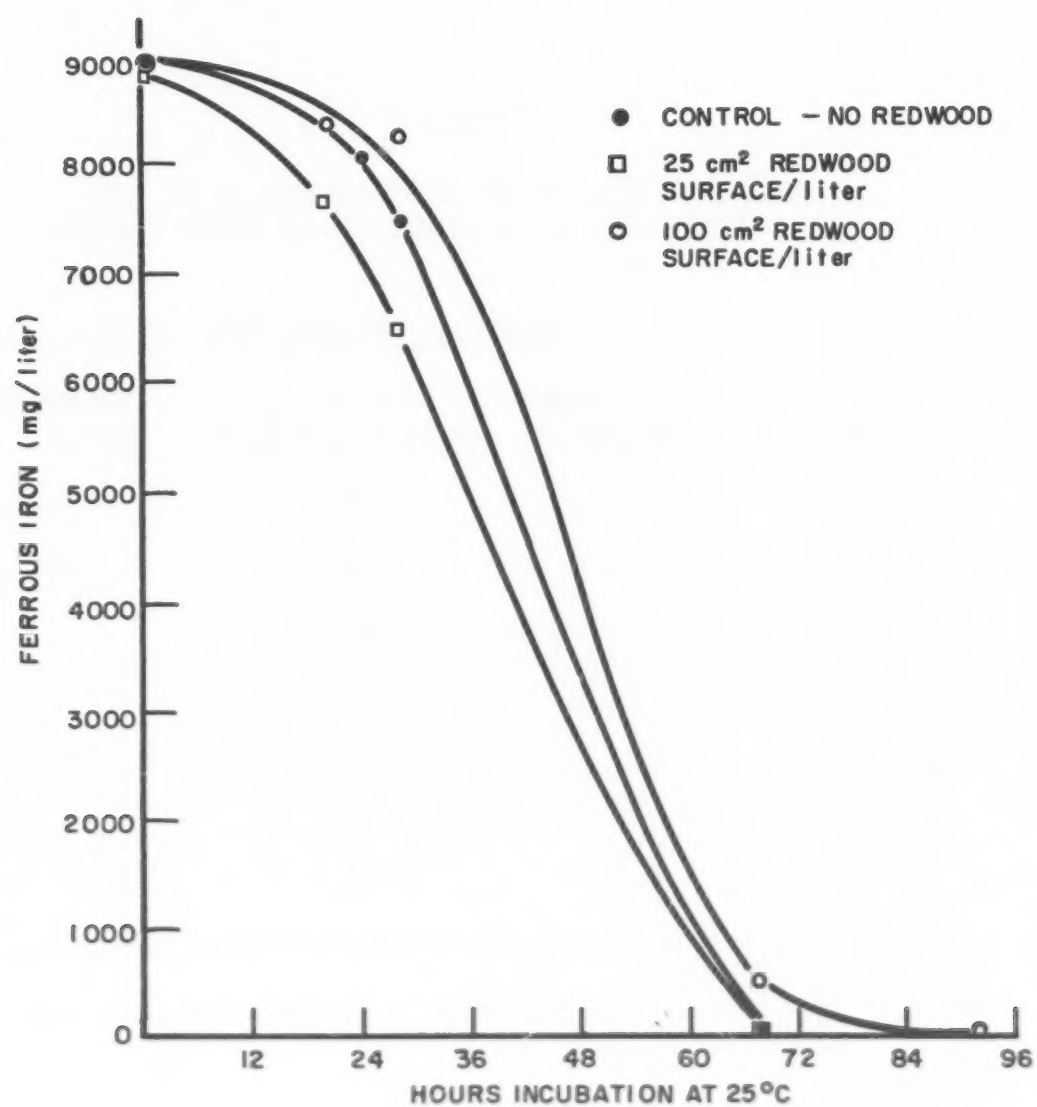


Figure 24 EFFECT OF UNLEACHED REDWOOD ON FERROUS OXIDATION
SOUTHWEST - I CULTURE, SHAKE FLASK STUDY

TABLE 7
 OXIDATION OF 9K MEDIUM BY CONTINUOUS CULTURE
 FERROUS IRON FEED - 9,000 mg/l
 CULTURE - Southwest-1 culture

Average Residence Time (hours)	Effluent Fe ⁺⁺ Concentration (mg/l)	Average Oxidation Rate (mg/l/hr)	Population (per ml)
87	81	102	10 ⁸
77	131	115	10 ⁸
43	163	205	10 ⁸
29	191	304	10 ⁸
25	217	351	10 ⁸
16	295	531	10 ⁸
12 (1st Day)	1049	662	10 ⁸
12 (4th Day)	2053	579	10 ⁸
12 (8th Day)	961	695	10 ⁸
12 (10th Day)	2500	542	10 ⁸
12 (12th Day)	5289	310	10 ⁸
32	165	276	10 ⁸
20	307	434	10 ⁸
14	474	609	10 ⁸
12 (1st Day)	629	710	10 ⁸
12 (4th Day)	1975	598	10 ⁸
12 (5th Day)	5624	294	10 ⁸

is about 14 hours. As expected, the rate of iron oxidation increased as residence time was decreased. At the 14-hour residence point, ferrous iron oxidation was occurring at about 600 mg/l/hr.

Increased aeration and/or addition of more ammonium and phosphate salts did not influence operation; therefore, it appears that iron oxidation was being limited by the metabolic capabilities of the bacterial cells present.

Operation at Lower Ferrous Iron Feed Concentrations

Using the same technique described for the 9K medium operation, the continuous feed system was used to examine 9K media modified to contain 1,000 mg/l or 500 mg/l ferrous iron. Results of these investigations are shown in Table 8. As may be seen, operation at lower ferrous iron influent concentrations allowed the concomitant use of lower residence times. As might be anticipated, lower rates of iron oxidation were experienced at these lower iron concentrations.

Aeration Rate

To insure that aeration rate was not limiting rate of iron oxidation, a rather extensive check was made during operation of the continuous feed system being fed ferrous iron at 500 mg/l. Aeration rates were decreased at two residence times: two hours and six hours. The resultant data is shown in Table 9. As may be seen, aeration at $1/3$ or $1/4$ VVM should not come close to limiting the rate of iron oxidation at iron concentrations substantially higher than any utilized in studies reported in this communication.

Carbon Dioxide Enrichment

In order to determine if insufficient carbon dioxide might be limiting the rate of iron bacterial growth, and thereby iron oxidation, the partial pressure of CO_2 in the sparger gas was increased. Because of the very low concentration of CO_2 relative to oxygen in air, it was possible to make large increases in the CO_2 content of air without significantly altering the percentage of oxygen present.

As may be seen in Table 10, the addition of about $1\frac{1}{2}$ order of magnitude more CO_2 to the microbial oxidation system had no significant effect on iron oxidation.

Multistaging of Oxidation Vessels

Effect on total efficiency of iron oxidation was examined by the addition of one or two oxidation vessels in series downstream of the original continuous feed oxidation tank. Although neither of these added vessels was quite as large as the first stage, it was still possible to develop a picture of the economics of such a setup. The results of this study are presented in Table 11.

TABLE 8
OXIDATION OF 1000 mg/l AND 500 mg/l FERROUS IRON BY CONTINUOUS CULTURE

Influent Fe ⁺⁺ Concentration (mg/l)	Average Residence Time (hours)	Effluent Fe ⁺⁺ Concentration (mg/l)	Average Oxidation Rate (mg/l/hr)	Population (per ml)
1000	8.0	48	119	10 ⁸
1000	5.9	56	160	10 ⁸
1000	4.2	68	222	10 ⁸
1000	2.1	118	420	10 ⁸
1000	1.6	200	500	10 ⁸
1000	0.95	400	624	10 ⁸
(operation at 0.95 hr residence time became progressively poorer)				
500	8.0	55	57	10 ⁸
500	6.0	78	70	10 ⁸
500	4.0	102	100	10 ⁸
500	3.0	105	135	10 ⁸
500	2.0	109	195	10 ⁸
500	1.8	120	210	10 ⁸
500	1.0	(operation became progressively poorer)		

TABLE 9
EFFECT OF AERATION ON THE MICROBIAL OXIDATION
OF FERROUS IRON
Steady State Values over Four-Day Intervals
Influent Ferrous Iron Concentrations of 500 mg/l

	<u>Aeration Rate (VVM)</u>	<u>Fe⁺⁺ Oxidation Rate (mg/l/hr)</u>
Average Residence of 2 Hours	1/4	185
	1/3	202
	1/6	215
	1/12	200
	1/15	192
	1/20	192
Average Residence of 6 Hours	1/4	75
	1/25	80
	1/30	71
	1/40	75
	1/80	73
	1/100	69
	1/200	40

TABLE 10
EFFECT OF CARBON DIOXIDE CONTENT OF
SPARGER GAS ON IRON OXIDATION

<u>% CO₂ in Sparger Gas</u>	<u>% O₂ in Sparger Gas</u>	<u>Fe⁺⁺ Oxidation Rate (mg/l/hr)</u>
0.03	21	48
0.7	21	42
3.2	20	47
6.2	19	44
16.7	17	40

TABLE 11

MULTISTAGE CONTINUOUS OXIDATION OF FERROUS IRON

Medium: 9K Medium Modified to Contain 1000 or 500
 mg/l Fe^{++}
 Aeration Rate: $1/3$ VVM
 Oxidation Vessel Sizes: 6000 ml, 4000 ml, 4000 ml

Fe^{++} Feed (mg/l)	Retention Time (hours)				Effluent Fe^{++} (mg/l)
	Stage I	Stage II	Stage III	Total	
1000	8	0	0	8	48
1000	4.8	3.2	0	8	34
1000	3.4	2.3	2.3	8	29
1000	5	0	0	5	65
1000	3	2	0	5	60
1000	2.1	1.4	1.4	4.9	42
500	6	0	0	6	78
500	3.6	2.4	0	6	46
500	2.6	1.7	1.7	6	38
500	4	0	0	4	102
500	2.4	1.6	0	4	58
500	1.8	1.2	1.2	4.2	38

Although the effects were not large, it may be observed that multistaging at equivalent total residence times does provide better quality effluents in terms of ferrous iron concentration. Apparently this effect is due to the presence of higher bacterial cell concentrations in Stages II and III than in Stage I presumably due to washout from Stage I. It was observed that the cell populations in the stages downstream of Stage I were about one order of magnitude larger than in Stage I.

Natural Mine Drainage Water as Substrate

Once the characteristics of microbial oxidation of ferrous iron were established in defined media, it became necessary to determine if the same patterns would extend to characteristic field waters. For these studies water from two coal mine discharges, Lynch Shaft and Core, were obtained and transported to the laboratory using appropriate precautions to minimize changes in the water during transit. Important chemical characteristics of these waters are given in Table 12. The data indicate that appropriate types and concentrations of nitrogen and phosphorus were present in the waters to adequately support iron bacterial growth. Initial operating conditions of the continuous feed oxidation system were selected that would most likely provide rapid biological activity and consisted of a residence time of six hours in Stage I, aeration at 1/5 VVM and a temperature of 25°C. The iron oxidation data thus obtained are presented in Table 13. By comparing oxidation rates in Table 13 with those in Table 8 using synthetic medium, it may be seen that the Core water was oxidized much more rapidly than the synthetic medium, while the Lynch Shaft water was oxidized at about the same rate as the synthetic medium.

Effect of Interrupted Aeration

The possibility of a mechanical failure always exists in a plant or process system operation. In order to simulate just what effect such an occurrence might produce, aeration to the continuous feed system was suddenly stopped while operating at a six-hour residence time in Stage I and a four-hour residence time in Stage II. The dissolved oxygen content dropped from 4.8 mg/l to 2.2 mg/l after 60 minutes, and the ferrous iron concentration in the oxidation vessels more than doubled within 100 minutes (Table 14). Without aeration, the system degenerated quite rapidly.

Limestone Neutralization

The necessity of converting ferrous iron to the ferric state prior to use of limestone to neutralize acidity and precipitate ferric hydroxide from mine drainage water has been discussed in an earlier portion of this report. However, it is questionable whether bio-oxidation of the iron needs to be carried to completion or if some significant quantity of ferrous iron could remain and limestone still be efficiently utilized to produce an acceptable treated water. This final treatment must provide

TABLE 12
CHEMICAL ANALYSIS OF MINE DRAINAGE WATERS

	<u>pH</u>	<u>Acidity (mg/l)</u>	<u>Fe⁺⁺ (mg/l)</u>	<u>Total Fe (mg/l)</u>	<u>P₂O₅ (mg/l)</u>	<u>NH₃ (mg/l)</u>
Core MDW*	3.3	3430	1535	1550	3.4	4.9
**	3.3		1506.6		20.0	15.0
Lynch Shaft*	2.45	3260	735	1130	1.1	2.4
**	2.8		660.7		47.0	13.0

* Analysis of MDW when shipped November 26, 1968

** Analysis of MDW when received December 2, 1968

TABLE 13
CONTINUOUS FEED STUDIES WITH NATURAL MINE WATERS

Stage I* - 6 Hours Retention				Stage II* - 4 Hours Retention			
Fe^{++} Feed (mg/l)	Fe^{++} in Effluent (mg/l)	Fe^{++} Oxidation Rate (mg/l/hr)	D.O.** (mg/l)	Fe^{++} Influent (mg/l)	Fe^{++} in Effluent (mg/l)	Fe^{++} Oxidation Rate (mg/l/hr)	D.O.** (mg/l)
Core MDW Feed							
1454.5	38.7	235.9	6.9	38.7	10.1	7.2	7.7
Lynch Shaft MDW Feed							
465.0	26.4	73.1	7.0	26.4	11.1	3.9	7.8

* Aeration rate 1/5 VVM - 1200 cc/min. Stage I, 800 cc/min. Stage II

**D.O. - Dissolved oxygen

TABLE 14

EFFECT OF NON-AERATION ON FERROUS OXIDATION DURING CONTINUOUS FEED

Non-Aeration Time (Min.)	Stage I - 6 Hours Residence			Stage II - 4 Hours Residence		
	Feed (mg/l Fe ⁺⁺)	Effluent (mg/l Fe ⁺⁺)	D.O. (mg/l)	Feed (mg/l Fe ⁺⁺)	Effluent (mg/l Fe ⁺⁺)	D.O. (mg/l)
0	437.5	49.1	4.8	49.1	8.9	6.8
20			3.6			6.4
60	435.3	78.1	2.2	78.1	8.9	6.2
100	432.8	104.9	0.4	104.9	15.6	5.4

effluent water containing not more than 7 mg total iron per liter, having a pH in the range of 6 to 9, and no un-neutralized acidity.

Effluent samples from Stage II of the continuous feed system were used for limestone neutralization studies in an attempt to clarify this uncertainty. Limestone rock dust with a calcium carbonate content of approximately eighty percent was used to neutralize partially oxidized effluents.

Stoichiometric quantities of rock dust required were calculated from the titratable acidity of each individual sample. These amounts of limestone were added to one-liter quantities of each effluent and the resultant slurries mixed for ten minutes with a magnetic stirrer. The neutralized liquors were then allowed to settle, and samples of the supernatants taken at subsequent times were analyzed for total iron contents. The data obtained from these experiments are presented in Table 15 and Figure 25. Significantly, it was found that waters containing as much as 89 mg ferrous iron per liter could be limestone neutralized to produce a water containing less than 7 mg/l total iron and meet the other criteria as well.

Effect of Added Solids during Limestone Neutralization

The previous experiments indicated that neutralized solids settled more rapidly in samples containing higher concentrations of total iron. Therefore, the possibility of decreasing settling time by the addition of iron solids prior to limestone treatment was investigated. Some precipitation of ferric iron always occurred in the first stage of the bio-oxidation system during normal operation. At low aeration rates there was insufficient agitation to carry all of these solids out of the oxidation vessels.

Solids from 500 ml of Stage I slurry were collected on Whatman No. 1 filter paper by vacuum filtration. The moisture content of the solids collected in this manner was determined to be approximately 65 weight percent. Solids were weighed and added to one liter of Stage II oxidized effluent. Analyses for total iron, ferrous iron, acidity and pH were performed and rock dust neutralization carried out. The data for two settling rate studies are presented in Table 16 and Figure 26. Also shown in Figure 26 is a control to which no iron solids were added. The effect of solids addition was quite significant in that the required settling time was decreased from 16 hours to 5 hours. Solids removal from the oxidation vessel in this manner with return of the filtrate to the oxidation vessel did not adversely effect bio-oxidation of ferrous iron in the oxidation vessel.

Sulfate-Reducing Bacteria

Effect of pH on Growth

Of the cultures listed in Table 2, none were found capable of growing or producing hydrogen sulfide at pH values of 5 or lower. Incubation was at 25°C and 35°C in Difco Sulfate API Broth adjusted to pH 2, 3, 4, 5, 6, 7

TABLE 15

EFFECT OF RESIDUAL FERROUS IRON ON LIMESTONE NEUTRALIZATION

Total Residence (hours)	Stage II Oxidized Liquor				Limestone Neutralized Liquor		
	Total Fe (mg/l)	Fe ⁺⁺ (mg/l)	pH	Acidity (mg/l CaCO ₃)	pH	Settling Time (hours)	Total Iron in Supernatant (mg/l)
5		85	2.4	2100	6.25	2 4 6 22.5	35.7 29.0 24.6 2.2
5.8	435	85	2.4	1900	6.2	2 4 6 23	42.4 27.2 26.8 6.7
6.7	487	49	2.5	1770	6.3	2 22	20.1 3.3
7.5	453	65	2.4	1750	6.5	1 17	15.6 6.7
7.5	469	89	2.4	1880	6.5	0.75 2.0 3.0 4.0 5.0 10.75 21.0	46.9 40.2 37.9 33.5 33.5 15.6 2.2

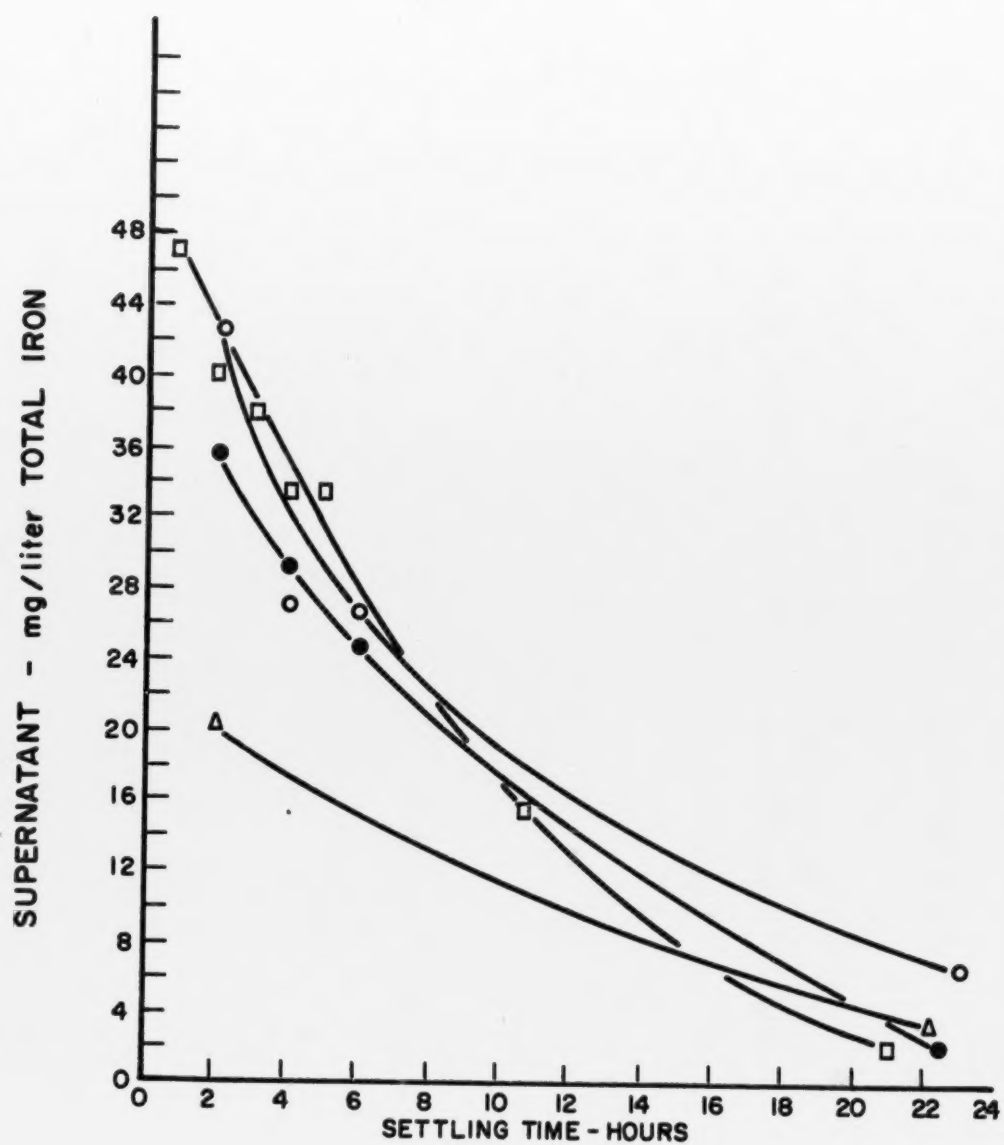


Figure 25 IRON PRECIPITATION FOLLOWING LIMESTONE NEUTRALIZATION OF PARTIALLY OXIDIZED MEDIUM.

TABLE 16

NEUTRALIZATION AND SETTLING STUDIES OF STAGE II OXIDIZED LIQUOR

Total Retention Time 7.5 Hours

Total Iron (mg/l)	Fe ⁺⁺ (mg/l)	pH	Acidity (mg/l CaCO ₃)	Neutralized pH	Settling Time (Hours)	Supernatant (mg/l Total Iron)	
453	65	2.4	1750	6.5	1	15.6	Study A Oxidized Liquor
					17	6.7	
	60	2.4	2020	6.1	1	6.7	Oxidized Liquor plus Stage I Solids (15.4 gms/l)*
					17	2.2	
469	89	2.4	1880	6.5	0.75	46.9	Study B Oxidized Liquor
					2.0	40.2	
					3.0	37.9	
					4.0	33.5	
					5.0	33.5	
					10.75	15.6	
					21.0	2.2	
2745	67	2.35	2920	6.15	0.75	15.6	Oxidized Liquor plus Stage I Solids (23.5 gms/l)*
					2.0	8.9	
					3.0	8.9	
					4.0	8.9	
					5.0	6.7	
					10.75	2.2	
					21.0	2.2	

* Wet weight of solids

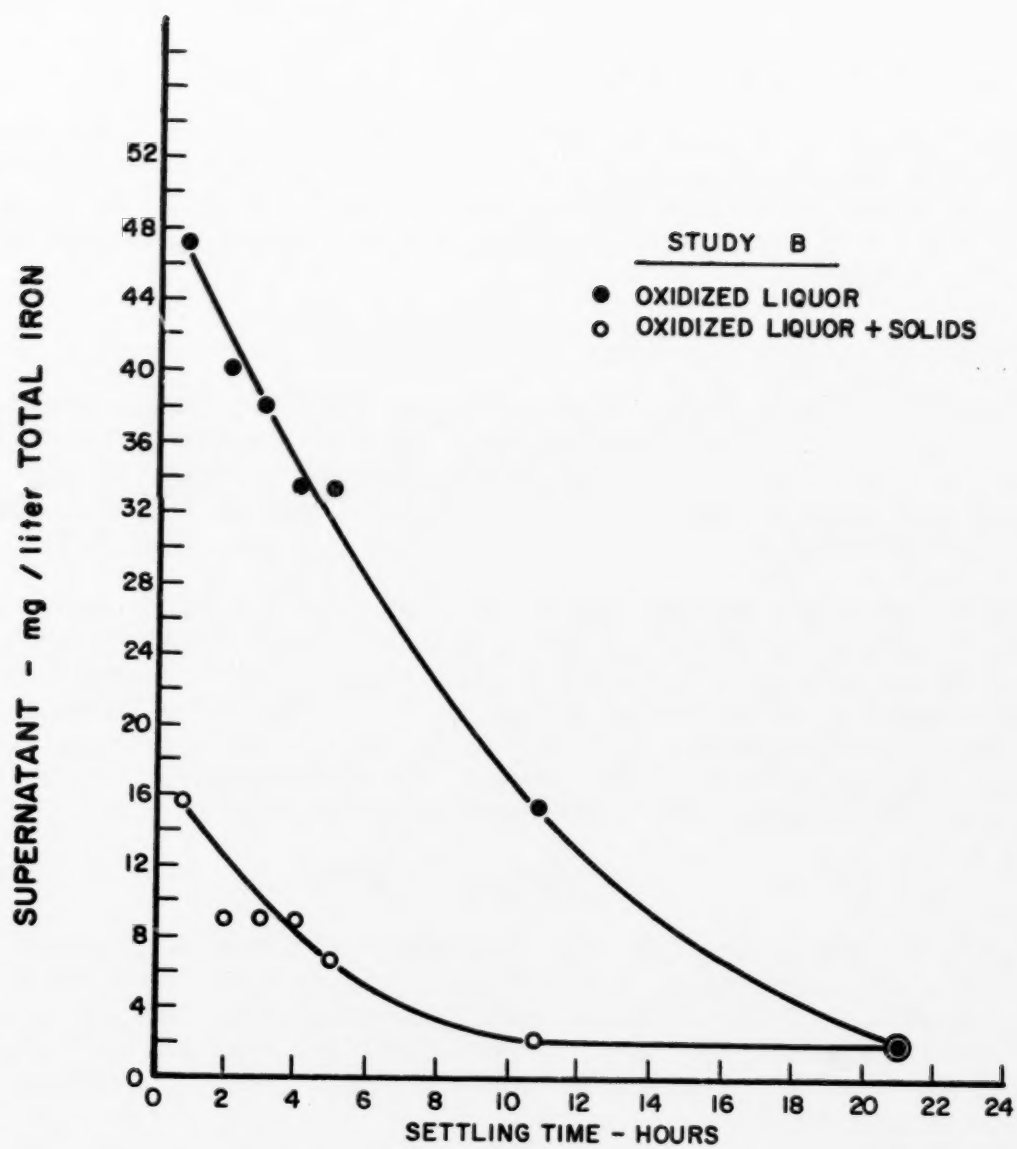


Figure 26 EFFECT OF ADDED SOLIDS ON LIMESTONE NEUTRALIZATION AND SETTLING OF STAGE II EFFLUENT

or 8 with KOH or HCl. The media were deaerated by autoclaving prior to inoculation with 28-hour old cultures growing at pH 7. Bottles were held for 30 days before discarding. All cultures grew and produced H_2S at pH 6, 7 and 8. Attempts to acclimate several of the field cultures to low pH by stepwise decrease of pH at half-unit intervals were unsuccessful.

Growth in Mine Drainage Water

Sodium lactate at 0.1 weight percent was added to four acid mine drainage waters and the resultant media inoculated with sulfate-reducer cultures isolated from these corresponding waters in the field. In no instance did growth or H_2S production occur. The API strain also refused to grow in these media.

Growth in Synthetic Media

As in the previous system, sodium lactate (0.1 weight percent) was used as the carbon source in 9K medium modified to contain 500 mg/l ferrous iron. pH adjustments were also made using KOH or HCl. Of six cultures tested, all grew at pH 6, but none grew or produced H_2S at lower pH values. Acclimation did result in growth at pH 5.5 but not at pH 5.0.

Using a platinum - calomel electrode system an effluent sample from the Stage II oxidation vessel of the continuous feed oxidation system was found to possess an Eh (oxidation-reduction potential) of +40 mv. An aliquot of this material was purged with H_2S until the Eh reached -115 mv. Following pH adjustments from 2.5 to 6.5 at 0.5-unit intervals, flasks of the reduced media were inoculated with the Southwest sulfate-reducer culture. As before, growth occurred no lower than pH 5.5.

Pilot Plant Studies

In order to field test the iron bacterial oxidation of ferrous iron in acid mine drainage water, a pilot plant was designed and constructed by the Consolidation Coal Company near Enterprise, West Virginia. The plant was constructed and housed in a small building near an acid mine water source that normally contained about 500 mg/l ferrous iron and very little ferric iron.

Physically the pilot plant consisted of two, 1,000-gallon tanks in series, a 50-gallon inoculum tank and facilities to aerate the tanks, heat the two, 1,000-gallon oxidation tanks and add phosphoric acid and/or ammonia to the acid mine water influent if necessary. A flow diagram of the plant is shown in Figure 27.

Temperature of liquor in the two oxidation vessels was controlled by four, 22-kilowatt immersion heaters located in each stage. Air was supplied by a centrifugal blower rated at 100 CFM and 6 PSIG. Aeration rates to the oxidation tanks could be read and controlled by means of flowmeters upstream of each tank.

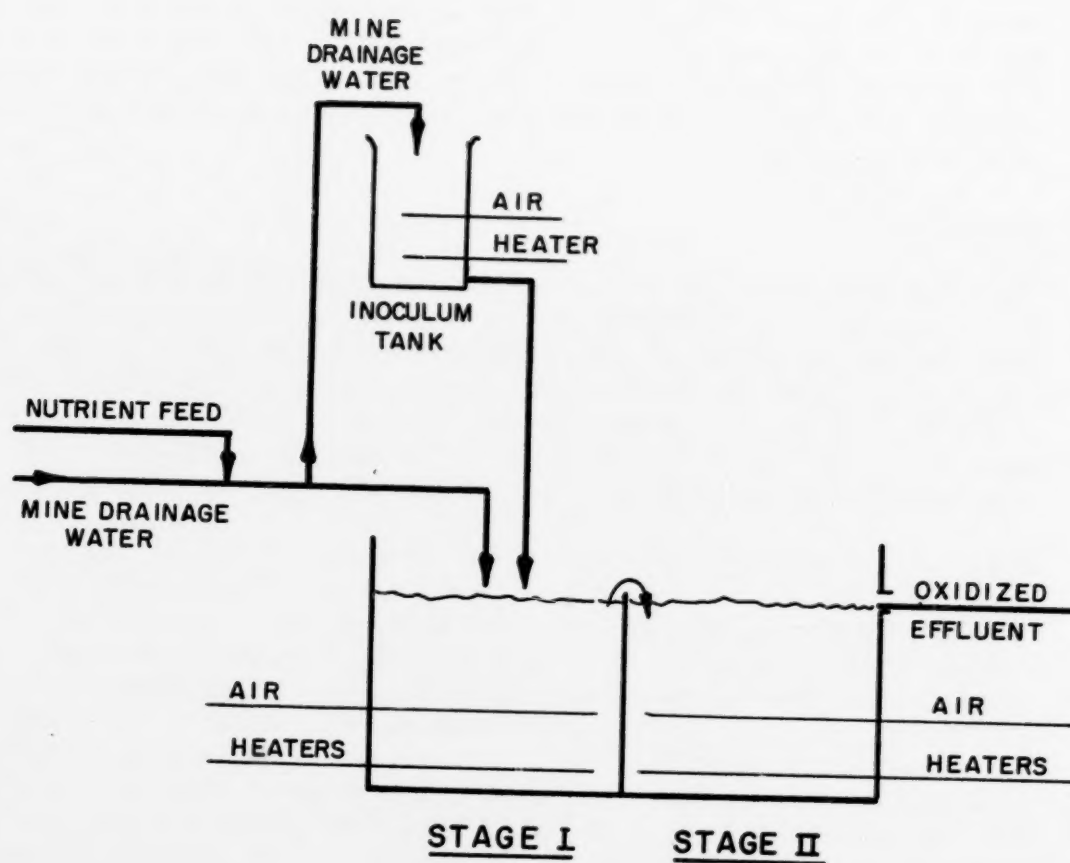


Figure 27 BIO-OXIDATION PILOT PLANT

The heated, aerated inoculum vessel was used to propagate an active iron bacterial culture for start-up as well as to maintain a constant supply of active culture in the event it might be needed during plant operation.

Mine drainage water was pumped from a mine to the surface and into a 10,000-gallon surge tank for minimizing flow and composition fluctuations. This supply water was pumped continuously to the bio-pilot plant at a rate of 10 to 12 GPM. A side stream was taken from this continuous flow as feed to the pilot plant. Post-oxidation treatment of the water was not piloted since this aspect of treatment has been relatively well covered in the literature and was somewhat outside the purpose of the overall investigation.

Start-Up

The inoculum tank was filled with acid mine water and inoculated with five gallons of laboratory culture grown in 9K medium. This mixed culture was composed of the cultures listed in Table 2 plus iron bacteria cultivated from the water to be used as feed to the pilot plant. Ferrous sulfate (1 g/l), anhydrous ammonia (20 mg/l) and phosphoric acid (20 mg/l) were added to the inoculum tank to insure rapid growth. The temperature was controlled at approximately 25°C, and the tank was aerated at 1/5 VVM.

When the iron bacterial population reached 10^8 /ml in the inoculum tank, the 1,000-gallon bio-oxidation vessels were filled with mine drainage water containing about 500 mg/l ferrous iron, aeration begun at 1/25 VVM, the temperature controlled at 26.5°C and the system inoculated. One day after inoculation the ferrous iron concentration had decreased to approximately 200 mg/l. At this point sufficient ferrous sulfate to raise the ferrous iron content back to 500 mg/l, 10 mg/l of ammonia and 20 mg/l phosphoric acid were added to the two oxidation vessels. This medium supplementation was subsequently done on a daily basis until the fourth day after inoculation when the bacterial population in the oxidation vessels reached 10^7 /ml.

Continuous Feed Operation

Based on previous laboratory experience, a 12-hour residence time, 1/25 VVM aeration and 26.5°C were selected as appropriate for good initial continuous feed operation.

Since the acid mine drainage water to be studied contained an average of 12 mg/l phosphate and 13 mg/l of ammonia nitrogen, these nutrients were not supplied. As might be expected of a field water, the iron content varied from time to time, but the variation was usually less than ± 100 mg/l from a 500 mg/l average ferrous iron level. Very little ferric iron was ever detected in the water. When continuous flow operation was initiated, temperature was checked at all extreme spots as well as central locations in the two oxidation tanks. Temperature uniformity at all locations indicated adequate tank mixing by the aeration system.

After steady-state iron oxidation was established, ferrous iron concentrations at these same locations in the tanks were found to be equally uniform verifying that the tanks were completely mixed.

Continuous feed oxidation at the 12-hour average residence time is shown in the table below:

TABLE 17

EFFECT OF FERROUS FEED VARIATION ON OXIDATION RATE

Average Residence Time: 12 hours
Operating Temperature: 26.5°C
Aeration Rate: 1/25 VVM

<u>MDW Feed</u> <u>mg Fe⁺⁺/l</u>	<u>Effluent</u> <u>Fe⁺⁺ Concentration</u> <u>(mg/l)</u>	<u>Oxidation Rate</u>
		<u>mg Fe⁺⁺/l/hr</u> <u>Stage I</u>
483	310	14
560	347	18

From these data it can be seen that a variation of about 80 mg/l in the influent produced only a minor change in oxidation rate. More significantly, oxidation rates at this influent ferrous iron concentration and residence time were disappointingly low.

When the aeration rate to the oxidation tanks was increased from 1/25 VVM to 1/10 VVM, the average oxidation rate at the 12-hour retention time rose from 16 mg/l/hr to 38 mg/l/hr. Although dissolved oxygen values in the oxidation tanks were around 8 mg/l at the 1/25 VVM aeration rate and previous laboratory data indicated that 1/25 VVM aeration was adequate, the increased aeration produced a striking increase in rate of ferrous iron oxidation.

Using the 1/10 VVM aeration rate, retention time in the oxidation tanks was decreased to 8 hours and steady-state operation established. Subsequently the residence time was dropped to 7 hours. Shorter residence times at 26.5°C were not investigated in order to be able to examine certain other pilot plant operational characteristics within the project time allocation.

If the data in Table 18 are compared to the laboratory data in Table 8, it is apparent that the pilot plant was operating about 15 percent poorer than the laboratory findings would predict.

Attempts to improve oxidation by further increasing the aeration rate, addition of phosphate and addition of ammonia proved fruitless. The mine

TABLE 18

EFFECT OF RESIDENCE TIME ON FERROUS OXIDATION

	Residence Time - Hours	Aeration Rate - VVM	MDW Feed mg Fe ⁺⁺ /l	Temperature Degree C	Stage I		Stage II	
					Residual mg Fe ⁺⁺ /l	Oxidation mg Fe ⁺⁺ /l/hr	Residual mg Fe ⁺⁺ /l	Oxidation mg Fe ⁺⁺ /l/hr
88	12	1/10	517	26.5	60	39	21	3
	8.0	1/10	530	26.5	147	48	9	17
	7.0	1/10	492	26.5	119	53	8	16

drainage feed was examined for the presence of organic carbon and heavy metals that might have deterred microbial activity, but none of these agents were detectable in the water.

The temperature of the oxidation tanks was then dropped to 20°C where oxidation was characterized. Further data were obtained at 11°C which represented the average temperature of the influent mine drainage water during the time of the year at which this study was conducted (January to February). Results of these studies are shown in Table 19.

Rates of oxidation at 20°C were lower than the rate attained at 26.5°C, and doubling the aeration rate only increased oxidation by 5 mg Fe⁺⁺/l/hour. The increase in oxidation rate at four hours over that at eight hours is almost directly proportional to the ferrous iron concentration in the mine drainage water feed.

Bench-Scale Studies Using Pilot Plant Feed Mine Drainage Water

In view of the somewhat poorer showing of the pilot plant operation in comparison to previously demonstrated microbial oxidation capabilities in the laboratory, the continuous feed system used in the laboratory was transported to the pilot plant site for studies on the fresh pilot plant feed water. This system was inoculated in the same manner as the pilot plant and operated on 9K medium modified to contain 500 mg/l of ferrous iron until rapid iron oxidation was taking place.

The medium was then altered by substituting pilot plant feed water for the distilled water in 9K medium and leaving the ferrous iron concentration at approximately 500 mg/l. This step was taken to positively determine if the field mine drainage water contained any components that might adversely effect iron oxidation. Steady-state operating conditions following substitution of this medium for the 9K medium containing 500 mg/l ferrous iron remained constant for 4 days indicating there were no inhibitory problems inherent to the pilot plant feed water. System operation utilizing this feed is shown in Table 20.

At this point the medium being fed to the unit was altered to the pilot plant feed water to which was added sufficient ammonium sulfate and dibasic potassium phosphate to equal concentrations of these agents in 9K medium. Again, no impairment in iron oxidation rates was detectable.

Finally, the influent to the bench-scale unit was altered to the unmodified pilot plant feed water. Resultant operation data are presented in Table 20. As may be observed from the table, the system oxidized ferrous iron at rates predictable from the earlier laboratory data.

From the data available, the only apparent difference between the bench-scale system and the pilot plant system might have been the surface-to-volume ratios. Although microscopic examination of samples taken from oxidation vessel surfaces as well as from central locations within the vessels indicated no detectable difference in microbial cell densities,

TABLE 19
EFFECT OF TEMPERATURE ON FERROUS OXIDATION

MDW Feed $\text{mg Fe}^{++}/\text{l}$	Average Residence Time - Hours	Aeration Rate - VVM	Temperature Degrees C	Oxidation Rate $\text{mg Fe}^{++}/\text{l/hr}$		$\text{mg Fe}^{++}/\text{l}$ Final Effluent
				Stage I	Stage II	
550	8	1/10	26.5	40	17	94
550	8	1/10	20	25	22	224
575	8	1/10	11	20	19	263
550	8	1/5	20	30	22	134
700	4	1/10	20	31	30	456
725	4	1/10	11	26	23	529

TABLE 20

EFFECT OF FEED WATER ON OXIDATION RATE

Residence Time - Hours		Aeration Rate - VVM	Feed Water mg Fe ⁺⁺ /l	Temperature Degrees C	Oxidation Rate mg Fe ⁺⁺ /l/hr	
Stage I	Stage II				Stage I	Stage II
<u>Modified MDW Feed</u>						
7.8	11.7	1/10	515	25	57	5
6.0	9.0	1/10	355	25	52	4
5.0	7.5	1/10	438	25	77	7
4.3	6.5	1/10	429	22	81	11
<u>Unmodified MDW Feed</u>						
4.3	6.5	1/10	421	22	80	11

surface-to-volume ratios of the pilot plant and bench-scale units were calculated. The resultant values (Table 21) perhaps offer an explanation for the wide discrepancies in efficiencies of the two systems.

TABLE 21

PILOT PLANT AND BENCH UNIT SURFACE-TO-VOLUME RATIOS

<u>Oxidation Vessel</u>	<u>Surface-to-Volume Ratio</u>
Pilot Plant	0.13:1
Bench Unit	0.82:1

SECTION VI

DISCUSSION

Iron Bacterial Treatment of Acid Mine Drainage Waters

Although the literature reveals a significant amount of knowledge about the kinetics of acidophilic iron bacterial oxidation of ferrous iron in synthetic media, there have been few real efforts to utilize the capabilities of these organisms in the treatment of acid ferrous mine discharge waters. In this study attention was focused on determining if these iron bacteria would function well in field mine drainage waters and on characterizing the nutritional and physical factors that would have important economic bearing on the iron bacterial oxidation of ferrous iron in mine drainage waters.

Bench-scale investigations revealed that acidophilic iron bacteria can perform equally well in synthetic media and field mine drainage waters. Based on nitrogen and phosphorus studies, these elements are present in appropriate forms and quantities in many acid mine discharges so that supplementation for maximal bacterial activity would not be necessary. Although aeration of the water to supply oxygen and carbon dioxide is essential for rapid microbial activity, it appears that aeration rates of around 1/100 VVM do not limit iron bacterial activity in waters containing up to 500 mg/l ferrous iron if the aeration technique results in efficient gas transfer to the water. In terms of aerobic microbial conversion operations, this aeration requirement is very low.

Since non-ferrous nutritional factors do not appear to be economically important in the microbial oxidation of ferrous iron in mine drainage waters, the most crucial economic factors are residence time and temperature. Based on the literature and the data presented in this communication, microbial oxidation of iron at very low pH values can be accomplished using an equal or lower average residence time than can air oxidation of lime-neutralized equivalent waters. Therefore, capital investment and operating cost requirements for detention and aeration should be similar for these two treatment techniques.

At mine drainage water temperatures below 20°C, microbial oxidation of ferrous iron is impaired. At 10°C efficiency appears to be about 30 to 40 percent of that at 20°C. Although it might be possible to find or develop a strain of iron bacteria that would function somewhat better at 10°C than the cultures that have been studied, it is not likely that such an effort would be very successful. At present it appears that an iron bacterial oxidation operation receiving water at 10°C must bear the burden of an increased residence time or the cost of heat to elevate the water temperature to about 20°C. The economic significance of this problem will depend greatly upon the specific location where water treatment is necessary and obviously upon the influent raw water temperature. Glover (21, 22) has reported that high aeration rates and solids recycle significantly aid microbial oxidation of iron in acid mine waters. While the

necessity of high aeration rates is not clear, solids recycle may be an important consideration. Although not reported in the previous sections of this report, indications of higher concentrations of microorganisms on or adjacent to solids in oxidation vessels were noted. This situation needs to be clarified for it might indicate that other types of microbial - mine drainage water contact systems should be investigated. Many new waste treatment techniques utilize physical configurations that provide large surface areas for microbial activity to take place.

Finally, the scale-up problems encountered in this study when attempts were made to evaluate bench data with data obtained in a 1,000-gallon pilot plant need to be thoroughly investigated. There is little doubt that the problems, once clarified, could be solved by simple pilot plant modifications.

Microbial Production of Hydrogen Sulfide in Mine Drainage Waters

Although sulfate-reducing bacteria do not appear capable of growing or producing hydrogen sulfide at pH values below 5.5, this factor should not rule out their potential application to the treatment of acid mine drainage waters. Since these organisms will grow in such waters at higher pH values, it might be feasible to utilize their hydrogen sulfide generating capability downstream of the neutralization step. It would, however, be essential that the oxygen content and oxidation-reduction potential of the water be kept very low.

A further complication is that the sulfate-reducing bacteria are not autotrophic and, therefore, require organic carbon for growth. Obviously, the provision of organic carbon to a mine drainage water would entail some expense; therefore, only a very cheap carbon source would be suitable. To supply such a carbon source at mine drainage treatment locations may prove difficult.

SECTION VII

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A significant objective of this project was to investigate the use of microbiological treatment to remove iron from mine drainage waters. Such research projects, intended to assist in the prevention of pollution of water by industry, are conducted under Section 6b of the Water Pollution Control Act, as amended. This project of EPA was conducted under the direction of the Pollution Control Analysis Section, Ernst P. Hall, Chief, Dr. James M. Shackelford, Project Manager, and Dr. James E. Moyer, Project Officer.

SECTION VIII

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1	Accession Number	2	Subject Field & Group	SELECTED WATER RESOURCES ABSTRACTS INPUT TRANSACTION FORM
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5	Organization	Continental Oil Company, Ponca City, Oklahoma Research and Development Department
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6	Title	Microbiological Treatment of Acid Mine Drainage Waters
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10	Author(s)	Whitesell, Louis B., Jr. Huddleston, Robert L. Allred, Ray C.	16	Project Designation	EPA, WQO Contract No. 14010 ENW
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22	Citation	
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25	Identifiers (Starred First)	*Acid Mine Water Treatment, Microbial Oxidation of Mine Water
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27	Abstract	Laboratory studies demonstrated that both pure cultures and fresh field cultures of acidophilic iron bacteria could readily oxidize ferrous iron in both synthetic and natural acid mine drainage waters. In the presence of adequate forms and amounts of oxygen, carbon dioxide, nitrogen and phosphorus, ferrous iron was oxidized at rates up to 600 mg/l/hr. Average oxidation rate was found to vary with the ferrous iron concentration in the water; i.e., the higher the ferrous iron content, the greater the oxidation rate. Approximate requirements of oxygen, carbon dioxide, nitrogen and phosphorus by the iron bacteria were established. Multistaging of oxidation vessels in series was found to produce a more effective microbial oxidation system than use of a single oxidation reservoir. Limestone neutralizations of partially oxidized acid mine waters showed that such waters containing up to 90 mg/l ferrous iron could be successfully neutralized and result in discharge waters containing ≤ 7 mg/l total iron. For reasons as yet undetermined, attempts to duplicate laboratory findings with a 2,000-gallon pilot plant were not completely successful. Although sulfate-reducing bacteria were isolated from all of nine acid mine discharges examined, attempts to grow the cultures or produce hydrogen sulfide at pH values below 5.5 were unsuccessful.
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Abstractor	Authors	Institution	Continental Oil Company
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